



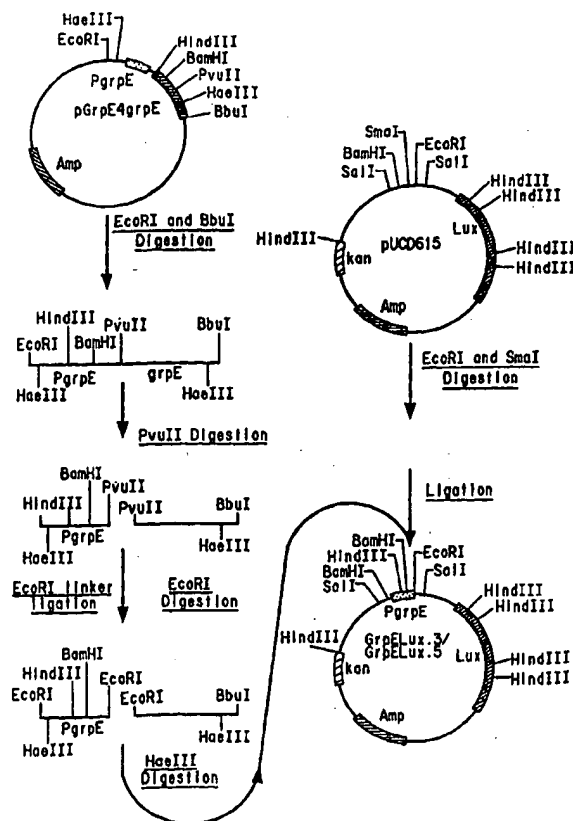
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US97/20793 (22) International Filing Date: 14 November 1997 (14.11.97) (30) Priority Data: 60/031,272                      15 November 1996 (15.11.96)      US (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LAROSSA, Robert, Alan [US/US]; 672 Kadar Drive, West Chester, PA 19382 (US). SCOLNIK, Pablo, Ariel [US/US]; 101 Bayview Drive, Chapel Hill, NC 27516 (US). (74) Agent: FLOYD, Linda, A.; E.I. du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: A SMALL VOLUME, HIGHLY SENSITIVE METHOD FOR DETECTING ENVIRONMENTAL INSULTS

## (57) Abstract

Subtle changes in environmental stress can now be detected and measured at sublethal levels as a generic response to environmental stress. The present invention provides an improved method to detect changes in the environmental stress level in very small volumes to permit very high throughput of analyses. The stress change is indicated as a change in the luminescence output of a genetically engineered microorganism. In the present invention, the luminescence gene complex is under the control of a stress inducible promoter. The volumes contemplated range from about 0.5 uL to about 10 mL of media in which the detector organism is suspended.



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TITLEA SMALL VOLUME, HIGHLY SENSITIVE METHOD  
FOR DETECTING ENVIRONMENTAL INSULTSFIELD OF INVENTION

5           The invention relates to an improvement in the detection of environmental  
insults at levels below those necessary to compromise cell metabolism. More  
specifically the invention provides a transformed bacterial host containing a DNA  
construct comprising a stress inducible promoter operably connected to a reporter  
gene or gene complex such that the presence of an environmental insult will  
10 induce the expression of the reporter genes. The preferred reporter genes are those  
that are responsible for bacterial bioluminescence. Volumes of between about  
1 uL to about 10 uL are useful, permitting high throughput analysis.

BACKGROUND

15           Increasing public concern and mounting government regulations have  
provided impetus for the development of environmental sensing systems capable  
of detecting contaminants in soil and ground water. Highly sensitive and specific  
detection systems incorporating analytical tools such as Gas Chromatography and  
Mass spectrophotometry have been known for several years; however, these  
systems require expensive equipment and skilled operators. Moreover, sample  
20 preparation and data analysis is often cumbersome and time consuming. Toxicity  
assays involving living organisms such as *Daphnia*, used in the standard U.S.  
water toxicity test, are simpler; however, these tests are non-specific and not  
particularly rapid.

          Somewhat more rapid are cell-based toxicity assays that incorporate a  
25 bacterial cell as the sensitive element. These systems use bacterial cells as a  
reagent in a conventional automated analytical system. For example the  
RODTox system (Central Kagaku., Tokyo, Japan) is a batch assay that measures  
bacterial oxygen consumption and was designed for use in sewage plants. Other  
bacteria based systems such as the GBI TOXALARM system (Genossenschaft  
30 Berliner Ingenieurkollektive, Berlin, Germany) can measure the presence of  
specific chemicals. The GBI TOXALARM is known to be able to detect the  
presence of as little as 0.1 ppm potassium cyanide in a sample. These detection  
systems are useful, but are hampered by cumbersome and complex detection  
systems. Recently, the phenomenon of bacterial bioluminescence has been  
35 regarded as providing a simpler and more sensitive mode of detection in  
environmental sensing systems.

          The phenomenon of bioluminescence first came under serious scientific  
scrutiny by Raphael Dubois in 1885 when he observed that the cell-free extracts  
obtained from the luminescent beetle *Pyrophorus* and the luminescent clam *Pholas*

gave a light emitting reaction *in vitro* when mixed at room temperature. Since that time bioluminescent systems have been identified and examined in a myriad of different organisms including the common firefly, marine coelenterates, fish, terrestrial and freshwater worms, as well as bacterial, algal and fungal species.

5        Bacterial bioluminescence is phenomenon in which the products of 5 structural genes (*luxA*, *luxB*, *luxC*, *luxD* and *luxE*) work in concert to produce light. The *luxD* product generates a C14 fatty acid from a precursor. The C14 fatty acid is activated in an ATP dependent reaction to an acyl-enzyme conjugate through the action of the *luxE* product which couples bacterial bioluminescence to  
10 the cellular energetic state. The acyl-enzyme (*luxE* product) serves as a transfer agent, donating the acyl group to the *luxC* product. The acyl-LuxC binary complex is then reduced in a reaction in which NADPH serves as an electron pair and proton donor reducing the acyl conjugate to the C14 aldehyde. This reaction couples the reducing power of the cell to bacterial light emission. The light  
15 production reaction, catalyzed by luciferase (the product of *luxA* and *luxB*), generates light. The energy for light emission is provided by the aldehyde to fatty acid conversion and FMNH<sub>2</sub> oxidation, providing another couple between light production and the cellular energy state.

      Recently, naturally bioluminescent organisms have been used as the  
20 sensitive element in toxicity tests. The MICROTOX system, (Microbics Corp., Carlsbad, CA) is an example. The MICROTOX system measures the natural baseline luminescence of *Photobacterium phosphoreum* and relates this to the hostility of the environment around the organism. Since the three couples, ATP level, NADPH level and FMNH<sub>2</sub> level, between light production and the central  
25 metabolic events of energy generation are necessary for light production in *Photobacterium phosphoreum*, any insult that interferes with the availability or interaction of these metabolites will cause a decrease in the activity of the bioluminescence(*lux*) system and therefore a related decrease in light production by the organism.

30        A main attribute of bioluminescent systems is that the decrease in light production is rapid, occurring within minutes of exposure to an insult. Another key advantage of these systems is that light detection can be exquisitely sensitive (down to the level of single photons), and is readily adaptable to portable field units. Furthermore, the logistics of light detection precludes the necessity of  
35 having the detector contact a wet, biological sample, which is a key weakness in competing technologies (such as ion-selective electrodes), where detector fouling and corrosion are responsible for significant down time.

      Recent advances in recombinant DNA technology have made it possible to express the luciferase (*lux*) gene complex as heterologous gene products. This is

generally accomplished by placing the *lux* structural gene complex under the control of a host promoter. So, for example cDNA encoding firefly luciferase has been expressed in *E. coli* under the control of the *lacZ* promoter (Tatsumi et al., Biochem. Biophys Acta., 1131, (2), pp 161-165, (1992)), and the *luxAB* fusion gene has been expressed in *Bacillus* at levels comparable to those achievable in *E. coli* by placing it under the control of the powerful *Pxyn* promoter (Jacobs et al., Mol. Gen. Genet., 230(1-2), pp 251-256, (1991)).

Alternate systems to the MICROTOX system have been developed using recombinant genetics to transform bacteria to be the light emitting element in the assay. Rychert et al. (*Environ. Toxic. Water Qual.*, 6 (4), pp 415-422, (1991)) have shown that recombinant *E. coli* harboring the plasmid, pJE202, that contain the *lux* gene complex, was sensitive to  $Zn^{2+}$ , ethidium bromide, sodium pentachlorophosphate,  $Cu^{2+}$  and 2,4-dichlorophenoxyacetic acid. Response in this assay was registered by a decrease in baseline light emitted by the transformed *E. coli*.

Although the MICROTOX and similar systems are useful, their sensitivity is limited to detecting levels of insults that kill or cripple the cell metabolically. To be detected by these systems, the insult must have reached a level high enough to either interfere with the central metabolism of the cell or to inactivate the Lux proteins.

Frequently it is necessary to be able to detect levels of insults at levels below those needed to affect cell metabolism. Such is the case in waste treatment facilities where lethal concentrations of pollutants can eradicate the useful microbial population, incurring significant cost and plant down time. A preferred sensing system would be one that would be able to detect the presence of insults at sublethal levels, before a useful microbial population could be harmed. Such an early warning could be used to trigger prompt remedial action to save the indigenous microbial population.

Recently, recombinant bacteria have been developed by fusing the *lux* structural genes to chemically responsive bacterial promoters and then placing such chimeras in appropriate hosts. These recombinant bacteria are sensor organisms that glow in response to specific stimuli. An example of this type of gene fusion is described by Burlage et al. (*J. Bacteriol.*, 172 (9) pp 4749-4757 (1990)) where a DNA fragment from plasmid NAH7 containing a promoter for the naphthalene degradation pathway was fused to the *lux* genes of *Vibrio fischeri* and used to transform a strain of *Pseudomonas*. The resulting transformant displayed an increase in light emission in the presence of naphthalene. The induction of bioluminescence was demonstrated to coincide with naphthalene degradation by the transformed organism.

Another test system specifically responsive to mercury (Hg) is described by H. Molders (EP 456667). Here, indicator bacterial strains are provided (by vector mediated gene transfer) containing a *mer* promoter, specifically inducible by Hg ions, fused to a bacterial luciferase (*lux AB*) genes complex which is responsible for bioluminescence. The test system of Molders relies on the induction of the *mer* promoter by the presence of mercury and the subsequent increase in light emission from the recombinant bacteria for the test results.

The methods of Burlage et al. and Molders offer several advantages over the art in that they specifically detect a single insult by the method of increased bioluminescence. These systems are useful for detecting the presence of specific chemicals in an environmental sample but are poor indicators of general cell toxicity. The promoter used by Burlage is functional in the naphthalene degradative pathway and is placed in a host that is able to use naphthalene as a carbon source. Hence this detection system is not associated with cell toxicity in any way. Similarly the *mer*-promoter of Molders is not indigenous to *E. coli* and therefore is not a native indicator of toxicity in *E. coli*. A more general test system for the primary detection of unknown insults would utilize a promoter specifically linked to cell toxicity or stress rather than one activated by one specific chemical.

Genes activated as a result of cellular stress provide an advantageous alternative strategy for the detection of environmental insults. Stress genes are found in all cells and are defined as those genes activated as a result of any type of stress that might alter the normal cellular metabolism. Environmental stresses often induce synthesis of an overlapping set of proteins. The most well recognized class of stress genes are the heat shock genes encoding a set of cellular proteins thought to have roles in refolding, recycling and resynthesis of proteins. The heat shock phenomenon was first described as a response to an increased temperature. Subsequent work has shown that exposure to a variety of stresses including phage infection, macrophage envelopment, as well as the presence of organic molecules and heavy metals can also trigger the heat shock response. The common theme of the inducing agents may be unfolding of some proteins within the cell. (LaRossa et al., *Mol. Microbiol.*, 5(3), pp 529-534, (1991)). Thus the response may integrate and report a wide range of environmental insults. VanBogelen et al. (*J. Bacteriol.*, 169(1), pp 26-32, (1987)) have demonstrated that a variety of chemicals are able to induce the heat shock genes in *E. coli*, including CdCl<sub>2</sub>, H<sub>2</sub>O<sub>2</sub>, ethanol, puromycin and nalidixic acid. Blom et al. (*Appl. Environ. Microbiol.*, 58(1), pp 331-334, (1992)) teach that the exposure of *E. coli* cultures to benzene, CdCl<sub>2</sub>, chlorpyrifos, 2,4-dichloraniline, dioctylphthalate, hexachlorobenzene, pentachlorophenol, trichloroethylene, and tetrapropyl-

benzosulfonate leads to the induction of up to 39 different stress proteins, as analyzed by two dimensional gel electrophoresis.

Since the cell attempts to maintain a steady state, stress responses are activated well below the minimal inhibitory concentration for any condition that serves as a triggering factor. This fact would make the use of stress responses in any environmental monitoring system particularly advantageous since detection of insults could be accomplished before microbial cell death. Such a system would be extremely useful in waste treatment facilities where environmental pollutants are often not detected until after the microbial population has been destroyed.

To date the induction of stress responses has been utilized in the area of environmental testing with only moderate success. Koehler et al. (*Arch. Environ. Contam. Toxicol.*, 22(3), 334-8, (1992)) describe a test system to assay the levels of HSP70 protein in various species of molluscs and slugs in response to the presence of heavy metals and pesticides. Although the system demonstrated increased levels of HSP70 in response to the presence of  $Pb^{2+}$ , the technique is cumbersome and lacks sensitivity. A more sophisticated technique described is by Saunders et al. (WO 9002947). The Saunders et al. technique involves detecting increased levels of HSP60 and HSP70 in organisms exposed to pollutants in an aqueous environment.

A highly sensitive biological test system employing a facile detection mechanism, able to detect a wide variety of insults at levels well below those needed to kill microbial populations was disclosed in PCT/US 93/11527. Potential uses include monitoring of air and water quality, agrichemical and pharmaceutical design, manufacturing and fermentation process control, process monitoring and toxicity screening. These applications may benefit many enterprises including the chemical, beverage, food and flavor, cosmetics, agricultural, environmental, regulatory and health care industries. Applicant's improvement, adapting the biological test of PCT/US 93/11527 to small volume, high throughput applications, is disclosed herein in Example 24.

#### SUMMARY OF THE INVENTION

The present invention provides an improved method for detecting the presence of an environmental insult comprising the steps of: culturing a suitable bioluminescent detector organism capable of responding to the presence of an environmental insult by a change in luminescence; exposing said detector organism to the presence of a sample suspected of containing an environmental insult; measuring the change in luminescence produced by the detector organism; and correlating said change in luminescence with the level of environmental insult present in said sample. The improvement is directed at the use of very small

volumes that are used in the method to suspend the detector organism. In particular, the improvement involves

(a) dispensing a solution containing a prokaryote detector organism into at least one container having a volume of about 0.5 ul to about 10 ul, said  
5 prokaryote detector organism being genetically engineered to contain an expressible heterologous luxCDABE gene complex under the control of a stress-inducible promoter sequence wherein the promoter is responsive to a global regulatory circuit;

(b) exposing the prokaryote detector organism to an environmental  
10 insult;

(c) using a luminescent detector to measure an increase in luminescence of the prokaryote detector organism. The method wherein the container is a well of a microtiter plate, and the luminescent detector is an x-ray film. The method wherein the prokaryote detector organism is in the log phase  
15 when exposed to the environmental insult.

The invention is an improvement to the method described in PCT/US 93/11527, the text of which is included in this application.

#### BRIEF DESCRIPTION OF THE DRAWINGS

##### SEQUENCE LISTING, AND BIOLOGICAL DEPOSITS

20 Figure 1 is an illustration of the construction of plasmid pGrpELux.3 and pGrpELux.5.

Figure 2 is an illustration of the construction of plasmid pRY006.

Figure 3 is an illustration of the construction of plasmid pRY001 and pYR002.

25 Figure 4 is a graphic representation of the increase in luminescence by TV1060 in response to the presence of ethanol.

Figure 5 is a graphic representation of the increase in luminescence by TV1060 in response to the presence of increasing concentrations of ethanol.

Figure 6a is a graphic representation of the increase in luminescence by  
30 WM1021 in response to the presence of varying concentrations of ethanol.

Figure 6b is a graphic representation of the increase in luminescence by WM1026 in response to the presence of varying concentrations of ethanol.

Figure 7 is a graphic representation of the relative sensitivities of *tolC*<sup>+</sup> and *tolC*<sup>-</sup> detector cells transformed with pGrpE.Lux.5 to pentachlorophenol.

35 The following strains were deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) (12301 Packlawn Drive, Rockville, MD 20852, U.S.A.):



Depositor Identification	ATCC No.	Date of Deposit
TV1060	69142	3 December 1992
TV1061	69315	13 May 1993
TV1076	69314	13 May 1993
WM1202	69313	13 May 1993
WM1021	69141	3 December 1992
WM1026	69143	3 December 1992
WM1302	69316	13 May 1993

The "ATCC No." is the accession number to cultures on deposit at the ATCC.

#### DETAILED DESCRIPTION OF THE INVENTION

5       The present invention provides a method for the detection of environmental insults at sublethal levels, incorporating a detector organism containing an expressible gene fusion between a stress inducible promoter and a structural gene resulting in expression of the *lux* genes.

10       Environmental insults capable of being detected by the detector organism of the present invention include a variety of organic and inorganic pollutants commonly found in industrial sites, waste streams and agricultural run-off. Such compounds include but are not limited to the polyaromatic hydrocarbons (PAH), the halogenated aromatics as well as a variety of heavy metals such as lead, cadmium, copper, zinc, and cobalt. Compounds demonstrated to be detected by  
15       the method of the present invention include atrazine, benzene, copper sulfate, 2,4-dichlorophenoxyacetic acid, ethanol, methanol, 2-nitrophenol, 4-nitrophenol, pentachlorophenol, phenol, toluene, dimethylsulfoxide, lead nitrate, cadmium chloride, sodium chloride, acetate, propionate, hydrogen peroxide, puromycin, mercury chloride, 2,4-dichloroaniline, propanol, butanol, isopropanol, methylene  
20       chloride, Triton X100, acrylamide, methyl viologen, mitomycin C, menadione, ethidium bromide, serine hydroxamate and xylene. Other environmental stresses detected were low phosphate levels, poor nitrogen source, poor carbon source and irradiation with ultraviolet light.

25       The following definitions are used herein and should be referred to for claim interpretation.

The terms "promoter" and "promoter region" refer to a sequence of DNA, usually upstream of (5' to) the protein coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct

site. Promoter sequences are necessary but not always sufficient to drive the expression of the gene.

A "fragment" constitutes a fraction of the DNA sequence of the particular region.

5 "Nucleic acid" refers to a molecule which can be single stranded or double stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. In bacteria and in higher plants, "deoxyribonucleic acid" (DNA) refers to the genetic material while "ribonucleic acid" (RNA) is involved in the translation of the information from DNA into proteins.

10 "Regulation" and "regulate" refer to the modulation of gene expression controlled by DNA sequence elements located primarily, but not exclusively upstream of (5' to) the transcription start of a gene. Regulation may result in an all or none response to a stimulation, or it may result in variations in the level of gene expression.

15 The term "coding sequence" refers to that portion of a gene encoding a protein, polypeptide, or a portion thereof, and excluding the regulatory sequences which drive the initiation of transcription. A coding sequence may be one normally found in the cell or it may be one not normally found in a cellular location wherein, it is introduced, in which case it is termed a heterologous gene.

20 The coding sequence may be a composite of segments derived from different sources, naturally occurring or synthetic.

The term "construction" or "construct" refers to a plasmid, virus, autonomously replicating sequence, phage or nucleotide sequence, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source,  
25 in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

30 The term "transformation" refers to the acquisition of new genes in a cell after the incorporation of nucleic acid.

The term, "operably linked" refers to the fusion of two fragments of DNA in a proper orientation and reading frame to be transcribed into functional RNA.

The term "expression" refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the  
35 expression, a DNA chain coding for the sequence of gene product is first transcribed to a complimentary RNA which is often a messenger RNA and, then, the thus transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

The term "translation initiation signal" refers to a unit of three nucleotides (codon) in a nucleic acid that specifies the initiation of protein synthesis.

The term "plasmid" as used herein refers to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and  
5 usually in the form of circular double-stranded DNA molecules.

The term "restriction endonuclease" refers to an enzyme which binds and cuts within a specific nucleotide sequence within double-stranded DNA.

The term "bioluminescence" refers to the phenomenon of light emission from any living organism.

10 The term "*lux*" refers to the *lux* structural genes which include *luxA*, *luxB*, *luxC*, *luxD* and *luxE* and which are responsible for the phenomenon of bacterial bioluminescence. A *lux* gene complex might include all of the independent *lux* genes, acting in concert, or any subset of the *lux* structural genes so long as *luxA* and *luxB* are part of the complex.

15 The term "stress" or "environmental stress" refers to the condition produced in a cell as the result of exposure to an environmental insult.

The term "insult" or "environmental insult" refers to any substance or environmental change that results in an alteration of normal cellular metabolism in a bacterial cell or population of cells. Environmental insults may include, but are  
20 not limited to, chemicals, environmental pollutants, heavy metals, changes in temperature, changes in pH as well as agents producing oxidative damage, DNA damage, anaerobiosis, changes in nitrate availability or pathogenesis.

The term "stress response" refers to the cellular response resulting in the induction of either detectable levels of stress proteins or in a state more tolerant to  
25 exposure to another insult or an increased dose of the environmental insult.

The term "stress protein" refers to any protein induced as a result of environmental stress or by the presence of an environmental insult. Typical stress proteins include, but are not limited to those encoded by the *Escherichia coli* genes *groEL*, *groES*, *dnaK*, *dnaJ*, *grpE*, *lon*, *lysU*, *rpoD*, *clpB*, *clpP*, *uspA*, *katG*,  
30 *uvrA*, *frdA*, *sodA*, *sodB*, *soi-28*, *narG*, *recA*, *xthA*, *his*, *lac*, *phoA*, *glnA* and *fabA*.

The term "stress gene" refers to any gene whose transcription is induced as a result of environmental stress or by the presence of an environmental insult. Typical *E. coli* stress genes include, but are not limited to *groEL*, *groES*, *dnaK*, *dnaJ*, *grpE*, *lon*, *lysU*, *rpoD*, *clpB*, *clpP*, *uspA*, *katG*, *uvrA*, *frdA*, *sodA*, *sodB*,  
35 *soi-28*, *narG*, *recA*, *xthA*, *his*, *lac*, *phoA*, *glnA*, *micF*, and *fabA*.

The term "heat shock gene" refers to any gene for which its synthesis is positively controlled by the structural gene encoding the sigma-32 protein (*rpoH*).

The term "stress inducible promoter" refers to any promoter capable of activating a stress gene and causing increased expression of the stress gene product.

5 The term "detector organism" refers to an organism which contains a gene fusion consisting of a stress inducible promoter fused to a structural gene and which is capable of expressing the *lux* gene products in response to an environmental insult. Typical detector organisms include but are not limited to bacteria.

10 The term "log phase" or "log phase growth" refers to cell cultures of detector organisms growing under conditions permitting the exponential multiplication of the detector cell number.

The term "Relative Light Unit" is abbreviated "RLU" and refers to a measure of light emission as measured by a luminometer, calibrated against an internal standard unique to the luminometer being used.

15 Host Cells for Construction of Detector Organisms

Host cells for the construction of detector organisms which are suitable in the present invention include any cell capable of expression of the *lux* gene fusion where prokaryotic cells are preferred and where members of the enteric class of bacteria are most preferred.

20 Enteric bacteria are members of the family *Enterobacteriaceae* and include such members as *Escherichia*, *Salmonella*, and *Shigella*. They are gram-negative straight rods, 0.3-1.0 X 1.0-6.0 mm, motile by peritrichous flagella (except for *Tatumella*) or nonmotile. They grow in the presence and absence of oxygen and grow well on peptone, meat extract, and (usually) MacConkey's  
25 media. Some grow on D-glucose as the sole source of carbon, whereas others require vitamins and/or mineral(s). They are chemoorganotrophic with respiratory and fermentative metabolism but are not halophilic. Acid and often visible gas is produced during fermentation of D-glucose, other carbohydrates, and polyhydroxyl alcohols. They are oxidase negative and, with the exception of  
30 *Shigella dysenteriae* 0 group 1 and *Xenorhabdus nematophilus*, catalase positive. Nitrate is reduced to nitrite (except by some strains of *Erwinia* and *Yersina*). The G + C content of DNA is 38-60 mol% ( $T_m$ , Bd). DNAs from species within most genera are at least 20% related to one another and to *Escherichia coli*, the type species of the family. Notable exceptions are species of *Yersina*, *Proteus*,  
35 *Providencia*, *Hafnia* and *Edwardsiella*, whose DNAs are 10-20% related to those of species from other genera. Except for *Erwinia chrysanthemi*, all species tested contain the enterobacterial common antigen (Bergey's Manual of Systematic Bacteriology, D. H. Bergey et al., Baltimore: Williams and Wilkins, 1984).

Particularly useful in the present invention are *E. coli* and members of the genus *Salmonella* and those enterics for which the *recA* gene and its upstream promoter region has been identified

#### Stress inducible Promoters

- 5 The present invention provides a stress inducible promoter sensitive to the presence of an environmental insult. Stress inducible promoters from both prokaryotic and eukaryotic cells may be used however promoters from bacteria are preferred and promoters from *E. coli* are most preferred. Suitable stress inducible promoters may be selected from, but are not limited to the list of genes
- 10 under the heading "responding genes" given in Table I, below:

TABLE I			
STIMULUS	REGULATORY GENE(S)	REGULATORY CIRCUIT	RESPONDING GENES*
Protein Damage <sup>a</sup>	<i>rpoH</i>	Heat Shock	<i>grpE, dnaK, lon, rpoD, groESL, lysU, htpE, htpG, htpI, htpK, clpP, clpB, htpN, htpO, htpX, etc.</i>
DNA Damage <sup>b</sup>	<i>lexA, recA</i>	SOS	<i>recA, uvrA, lexA, umuDC, uvrA, uvrB, uvrC, sulA, recN, uvrD, ruv, dinA, dinB, dinD, dinF etc.</i>
Oxidative Damage <sup>c</sup>	<i>oxyR</i>	Hydrogen Peroxide	<i>katG, ahp, etc.</i>
Oxidative Damage <sup>d</sup>	<i>soxRS</i>	Superoxide	<i>micF, sodA, nfo, zwf, soi, etc.</i>
Membrane Damage <sup>e</sup>	<i>fadR</i>	Fatty Acid Starvation	<i>fabA</i>
Any <sup>f</sup>	?	Universal Stress	<i>uspA</i>
Stationary Phase <sup>g</sup>	<i>rpoS</i>	Resting State	<i>xthA, katE, appA, mcc, bolA, osmB, treA, otsAB, cyxAB, glgS, dps, csg, etc.</i>
Amino Acid Starvation <sup>h</sup>	<i>relA, spoT</i>	Stringent	<i>his, ilvBN, ilvGMED, thrABC, etc.</i>
Carbon Starvation <sup>i</sup>	<i>cya, crp</i>	Catabolite Activation	<i>lac, mal, gal, ara, tna, dsd, hut, etc.</i>

STIMULUS	REGULATORY GENE(S)	REGULATORY CIRCUIT	RESPONDING GENES*
Phosphate Starvation <sup>j</sup>	<i>phoB, phoM, phoR, phoU</i>	P Utilization	<i>phoA, phoBR, phoE, phoS, aphA, himA, pepN, ugpAB, psiD, psiE, psiF, psiK, psiG, psiI, psiJ, psiN, psiR, psiH, phiL, phiO, etc.</i>
Nitrogen Starvation <sup>k</sup>	<i>glnB, glnD, glnG, glnL</i>	N Utilization	<i>glnA, hut, etc.</i>

\* Genes whose expression is increased by the corresponding stimulus and whose expression is controlled by the corresponding regulatory gene(s).

- <sup>a</sup> Neidhardt and van Bogelen in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 1334-1345, American Society of Microbiology, Washington, DC (1987))
- <sup>b</sup> Walker in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 1346-1357, American Society of Microbiology, Washington, DC (1987))
- <sup>c</sup> Christman et al. *Cell* 41: 753-762 (1985); Storz et al. *Science* 248: 189-194 (1990); Demple, *Ann. Rev. Genet.* 25: 315-337 (1991)
- <sup>d</sup> Demple, *Ann. Rev. Genet.* 25: 31 337 (1991)
- <sup>e</sup> Magnuson et al. *Microbiol. Rev* 57: 522-542 (1993)
- <sup>f</sup> Nystrom and Neidhardt, *J. Bacteriol.* 175: 2949-2956 (1993); Nystrom and Neidhardt (Mol. Microbiol. 6: 3187-3198 (1992)
- <sup>g</sup> Kolter et al. *Ann. Rev. Microbiol.* 47: 855-874 (1993)
- <sup>h</sup> Cashel and Rudd in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 1410-1438, American Society of Microbiology, Washington, DC (1987)); Winkler in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 395-411, American Society of Microbiology, Washington, DC (1987))
- <sup>i</sup> Neidhardt, Ingraham and Schaecter. *Physiology of the Bacterial Cell: A Molecular Approach*, Sinauer Associates, Sunderland, MA (1990), pp 351-388; Magasanik and Neidhardt in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 1318-1325, American Society of Microbiology, Washington, DC (1987))
- <sup>j</sup> Wanner in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 1326-1333, American Society of Microbiology, Washington, DC (1987))
- <sup>k</sup> Rietzer and Magasanik in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C., et al. Eds., pp. 1302-1320, American Society of Microbiology, Washington, DC (1987)); Neidhardt, Ingraham and Schaecter. *Physiology of the Bacterial Cell: A Molecular Approach*, Sinauer Associates, Sunderland, MA (1990), pp 351-388

Table I indicates the relationship of responding gene(s) with a particular regulatory gene(s) and a regulatory circuit and the associated cellular stress response triggered by a particular stimulus.

Although the majority of the stress genes listed above in Table I are known to be positively regulated, the SOS response, produced as a result of DNA damage, represents a negatively regulated circuit. For definition of positive and negative control mechanisms see Beckwith in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1439-1443, American Society of Microbiology, Washington, DC (1987)).

The SOS response to DNA damage is well understood in *E. coli*. The product of the *lexA* gene (the LexA repressor) binds to operator elements controlling the expression of at least 17 chromosomal genes (Walker in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1346-1357, American Society of Microbiology, Washington, DC (1987)). Upon DNA damage or interference with DNA replication, an unknown SOS-inducing signal is produced. This signal interacts with the *recA* gene product converting it into a form that increases the rate of proteolysis of a limited number of repressor molecules (Little, *J. Bacteriol*, 175:4943-4950). These repressor molecules are the products of the chromosomal *lexA* gene and repressors encoded by and expressed from ultraviolet light-inducible phage genomes in the lysogenic state. SOS promoters are released from repression by RecA protein-mediated proteolysis of the LexA repressor. Among the SOS responsive promoters are *recA* and *uvrA*. It was seen that the *recA* promoter-*lux* fusion on a multicopy plasmid produced bioluminescence and resulted in a transformed host cell increasing bioluminescence in response to DNA damage. Although the precise mechanism of this result is not known it is clear that the invention, is not limited to the study of positively regulated global regulatory circuits because (1) some negatively regulated circuits will operate with promoters of responding genes in the multicopy state and (2) several means to place negatively controlled promoters in a single copy state exist [e.g., Oden et al., *Gene* 96:29-36 (1990); Symons et al., *Gene* 53:85-96 (1987); Winans et al., *J. Bacteriol*, 161:1219-1221 (1985); Arps and Winkler, *J. Bacteriol*, 169:1061-1070 (1987); Jasin and Schimmel, *J. Bacteriol*, 159:783-786 (1984)].

#### Construction of Expression Vectors and Transformed Hosts

The invention also provides a transformation vector containing a stress inducible promoter-*lux* gene fusion, capable of transforming a bacterial host cell for the expression of the Lux proteins. A variety of transformation vectors may be used, however, those capable of transforming *E. coli* are preferred. pGrpELux.3, pGrpELux.5, pRY001, pRY002, and pRY006 are five specific examples of

suitable transformation vectors whose construction is given in detail in the following text. These vectors represent only a sample of the total number of vectors created for the purpose of introducing stress promoter-*lux* reporter fusions into host cells. However, it will be readily apparent to one of skill in the art of molecular biology that the methods and materials used in their construction are representative of all other vectors described. Other preferred vectors are listed in Table V of Example 10.

pGrpELux.3 and pGrpELux.5 are vectors containing the *grpE* promoter while pRY001, pRY002 and pRY006 contain the *dnaK* promoter. pGrpELux.3, pGrpELux.5, and pRY006 were all created by the method of direct cloning while PCR protocols were employed as part of the construction method for pRY001 and pRY002. Transformation vectors such as these are common and construction of a suitable vector may be accomplished by means well known in the art. The preferred source of the *lux* genes is a pre-existing plasmid, containing a promoterless *lux* gene complex. Similarly, preferred sources of the stress inducible promoter DNA for the construction of the transformation vector are either also a pre-existing plasmid, where the stress inducible promoter DNA is flanked by convenient restriction sites, suitable for isolation by restriction enzyme digestion, or the product of a PCR reaction.

The pGrpELux.3 and pGrpELux.5, vectors are constructed from the *E. coli* stress gene *grpE*, and the *lux* gene complex. pGrpE4 is an *E. coli* vector derived from pUC18 (Pharmacia, Cat. No. 27-4949-01). pGrpE4 contains the *grpE* gene, including its promoter, bounded at the 5' end by an EcoRI site and at the 3' end by a BbuI site. Additionally, the *grpE* promoter is bounded at the 3' end by a PvuII site and an HaeIII site just downstream of the EcoRI site (Figure 1). Digestion with EcoRI and BbuI restriction enzymes yields a 1.1 kb fragment which corresponds to the *grpE* gene. Further digestion with PvuII produces two fragments, one of which contains the *grpE* promoter. The 3' PvuII site on the *grpE* promoter fragment is converted to an EcoRI site via ligation to phosphorylated EcoRI linkers. Further digestion by HaeIII yields a *grpE* promoter fragment conveniently bounded by a 5' HaeIII site and a 3' PvuII site (Figure 1).

The pUCD615 plasmid containing the *lux* gene complex is fully described by Rogowsky et al. (*J. Bacteriol.*, 169 (11) pp 5101-512, (1987)). Plasmid pUCD615 is a 17.6 kb plasmid which contains the genes for kanamycin and ampicillin resistance and contains the promoterless *lux* gene operon (Figure 1). pUCD615 is first digested with restriction enzymes EcoRI and SmaI, opening the plasmid, followed by ligation with the DNA fragments from the HaeIII digestion of pGrpE IV.



Typically, the products of the ligation reactions are screened by first transforming a suitable host and screening for bioluminescence. A variety of hosts may be used where hosts having high transformation frequencies are preferred. XL1Blue (Stratagene, LaJolla, CA) and DH5- $\alpha$  (GIBCO-BRL, Gaithersburg, MD) are two such hosts. Preferred methods of bioluminescence screening involve exposing gridded cultures of transformants to a suitable X-ray film, followed by visual analysis of the developed films for evidence of exposure. Reisolation of the plasmid from the transformed host and restriction digests followed by gel electrophoresis is used to confirm the existence of the correct plasmid. The plasmids pGrpELux.3 and pGrpELux.5, isolated from two different transformed colonies, are indistinguishable on the basis of restriction enzyme analysis. Under some experimental conditions cells transformed with pGrpELux.5 exhibited higher baseline bioluminescence than those transformed with pGrpELux.3 and hence pGrpELux.5 is preferred for the detection of many environmental insults.

The present invention further provides a transformed host cell capable of increased luminescence in the presence of an environmental insult. Many suitable hosts are available where *E. coli* is preferred and the *E. coli* strain RFM443 is most preferred. RFM443 is derived from W3102 which is fully described by B. Bachmann, in *E. coli and Salmonella typhimurium: Cellular and Molecular Biology* (Niedhardt et al. Eds., pp 1190-1220, American Society of Microbiology, Washington, D.C. (1987)). Transformation of RFM443 by pGrpELux.3 gives the new strain TV1060 which has been deposited with the ATCC under the terms of the Budapest Treaty. Transformation of RFM443 by pGrpELux.5 gives the new strain TV1061. The baseline of bioluminescence from strain TV1061 is greater than that from strain TV1060. *E. coli* TV1060 has been assigned ATCC No. 69142, and TV1061 has been assigned ATCC No. 69315.

The construction of the plasmid pRY006 containing the *dnaK* promoter followed a similar protocol to that of pGrpELux.3. DNA encoding the *dnaK* promoter was obtained from the Lambda phage 9E4 by digestion with the restriction enzymes EcoRI and BamHI. 9E4 is fully described by Kohara et al (Cell 50, 495-508, 1987) herein incorporated by reference. Restriction enzyme digestion produced a 3.7 kb DNA fragment encompassing the *dnaK* promoter region bounded on the 5' end by a BamHI site and on the 3' end by an EcoRI site. As in the construction of pGrpELux.3, the source of the *lux* gene complex is pUCD615. pUCD615 was first digested with BamHI and EcoRI restriction enzymes followed by ligation with the *dnaK* promoter fragments to produce the plasmid pRY006 (Figure 2).

Construction of pRY001 and pRY002 is similar to that of pRY006 except that PCR protocols were used to amplify the DNA encoding the *dnaK* promoter from 9E4. Briefly, PCR amplification of the *dnaK* promoter from 9E4 was accomplished using the *dnaK* promoter sequence as described by Cowing et al,  
5 PNAS 82, 2679-2683, 1985 herein incorporated by reference.

Amplification was carried out as described by the manufacturer (Geneamp PCR Reagent Kit, Perkin-Elmer Cetus, Norwalk, CT), herein incorporated by reference. The amplified product corresponding to the *dnaK* promoter region contained convenient BamHI and EcoRI sites determined by the construction of  
10 the amplification primers. The *dnaK* promoter region was ligated to pUCD615, previously digested with restriction enzymes BamHI and EcoRI.

Ligated DNA was used to transform *E. coli* strain DH5 $\alpha$  and the resulting transformants were screened for bioluminescence by exposure to X-ray film, and by restriction digests followed by analysis on agarose gels. The strain DH5- $\alpha$  was  
15 chosen for this initial screening due to its high transformation frequency. Two independent colonies were chosen. The two plasmids isolated from these transformants, pRY001 and pRY002, although isolated from independent colonies, are indistinguishable on the basis of restriction enzyme analysis and for the purposes of the present invention are considered identical. Under some  
20 experimental conditions cells transformed with pRY002 exhibited higher bioluminescence in response to environmental insults than those transformed with pRY001 and hence pRY002 is preferred for detection. pRY001, pRY002 and pRY006 were then used to transform RFM443 to create *E. coli* stain WM1021, WM1202 and WM1026 respectively. *E. coli* WM1021, WM1202 and WM1026  
25 have been deposited with the ATCC under the terms of the Budapest Treaty. *E. coli* WM1021 has been assigned ATCC No. 69141. *E. coli* WM1202 has been assigned ATCC No. 69313. *E. coli* WM1026 has been assigned ATCC No. 69143. As mentioned above, construction of the promoters of other stress genes fused to the lux reporter was identical to the construction of pRY001 and pRY002  
30 with the exception that the PCR primers and source of template DNA were different as dictated by the sequences of the promoters. The sequences of all of the promoters are published and are readily available through the Genbank database of nucleic acid sequences.

The stress inducible promoter-*lux* plasmid exists in the transformed host of  
35 the present invention as an autonomously replicating plasmid; however, the routiner will recognize that it is also possible to provide a transformed host wherein the stress inducible promoter-*lux* plasmid is integrated into the genome of the transformed host. This may be accomplished by means known to those skilled in the art. The stress inducible promoter may drive expression of a gene product

which in turn activates expression of the *lux* gene complex. In this case the promoter and *lux* gene complex might occur on different genetic elements.

As examples, any of a number of suppression mechanisms may be invoked [Hartman and Roth, *Advances in Genetics*, 17:1-105 (1973)]. In one, a chromosomally-integrated *lux* gene complex contains a nonsense mutation in either *luxC*, *luxD*, *luxA*, *luxB* or *luxE* and is driven by a constitutive promoter. The stress inducible promoter is fused such that it drives the expression of a nonsense suppressor gene. In the absence of stress, bioluminescence is not observed due to organisms' inability to synthesize the five requisite *Lux* proteins. If the organism is stressed, the suppressor gene is transcribed. Expression of the suppressor gene product allows expression of the five requisite *Lux* proteins and hence the organism produces light.

#### Membrane Mutations

Detector cells of the present invention optionally may contain mutations that will facilitate the screening process. Thus, the chemical of interest must be able to enter the cell, be retained in the cell, and interact with target molecules of the cellular machinery. Various mutations of *E. coli* are known to affect permeation into and accumulation within the cell. Strains carrying mutant alleles of *rfa* (Ames, B. N., F.D. Lee, and W. E. Durston, *Proc. Nat. Acad. Sci. USA*, 70(3): p. 782-786, (1973)), *envA* (Young, K. and L. L. Silver, *J. Bacteriol.*, 173: p. 3609-3614, (1991)), *imp* (Sampson, B. A., R. Misra, and S. Benson, *Genetics*, 122: p. 491-501, (1989)), *lpp*, (Giam, C. Z., S. Hayashi, and H. C. Wu, *J. Biol. Chem.*, (259): p. 5601-5605, (1984)) or *surA* (Tormo, A., M. Almiron, and R. Kolter, *J. Bacteriol.*, (172): p. 4339-4347, (1990)) have increased sensitivity to a variety of chemicals. Destruction of efflux pumps, with mutations such as *emr* (Ma, D., et al., *Mol. Microbiol.*, 16: p. 45-55, (1995)), or *acrAB* (Paulsen, I.T., et al., *Mol. Micro.*, 19: p. 1167-1175, (1996)), or the channels they use, with mutations such as *tolC* (Schnaitman et al., *J. Bacteriol.*, 172 (9), pp 5511-5513, (1990)), also result in increased chemical sensitivity. An appropriate host strain of *E. coli* or other bacteria may be constructed to carry a known mutation or combinations of mutations. Optionally, it is within the scope of the present invention to provide a detector organism containing the *tolC*<sup>-</sup> mutation, or other mutations that alter the characteristics of the cell membrane to facilitate the screening process.

Specifically, in order to create a highly sensitive detector organism with enhanced cell envelope permeability to toxic organics, a *tolC*<sup>-</sup> mutation was introduced into *E. coli* strain RFM443. The *E. coli* transductant DE112 is isogenic to strain RFM443 except for the mutation at the *tolC* locus. It was constructed by phage P1 mediated generalized transduction using a lysate grown on strain

CS1562 (*tolC::miniTn10*) (Schnaitman et al. *J. Bacteriol.*, 172 (9), pp 5511-5513, (1990)) as a donor and strain RFM443 as a recipient. Resultant tetracycline resistant transductants were screened for hypersensitivity to the hydrophobic compound crystal violet.

- 5 DE112 was transformed with either pGrpELux.5 or pRY002 according to standard transformation methods as described above to create the detector organisms TV1076 (*grpE lux fusion*) and WM1302 (*dnaK lux fusion*) containing the *tolC*<sup>-</sup> mutation. TV1076 and WM1302 have been deposited with the ATCC under the terms of the Budapest treaty and are designated ATCC No. 69314 and ATCC No. 69316 respectively.

10 Small Volume Assay Methods

- As has been mentioned, a variety of agents and conditions are known to produce a stress response in bacterial organisms. Modifications in assay systems, such as the reduction in culture media volume to allow for high throughput assay systems, may place a limitation on these assays where these conditions may also  
 15 produce a cell stress response. For example, small volume cultures (1-2 ul) are subject to high rates of evaporation (altering osmolarity and pH) as well as physical stress on the cell membrane produced by interaction of the cell with the surface of the medium. It has been shown for example that HeLa cells stressed in a hypertonic environment demonstrated in a rapid fall in cell volume, a 30%  
 20 decrease in cell viability as well as ultrastructural changes (Wheatley et al., *Mol. Physiol.* (1984), 6(3-4), 163-81).

- It is well known that the effect of osmotic stress on eukaryotic cells results in the induction of a variety of stress genes. It has been demonstrated for example that HSP-72 and HSP-25 in mammalian kidney cells (Rauchman et al., *Am. J.*  
 25 *Physiol.* (1997), 273(1, Pt. 2), F9-F17; Schober et al., *Pfluegers Arch.* (1997), 434(3), 292-299; Ohno et al., *Kidney Int., Suppl.* (1996), 57, S110-S118); HSP-70 in human HeLa cells (Hatayama, et al., *Biol. Pharm. Bull.* (1997), 20(6), 605-612); and thermo-tolerance in *Saccharomyces sp.* (Piper, P., *FEMS Microbiol. Rev.* (1993), 11(4), 339-56) are all induced by increased osmotic stress. Similarly,  
 30 osmotic pressure on prokaryotic species are known to influence the expression of heat shock and other stress induced proteins. For example, the bacterial global regulator sigma s has been implicated in the osmotic control of gene expression in *E. coli* (Hengge-Aronis, R., *Mol. Microbiol.* (1996), 21(5), 887-893; Chuang, et al., *J. Bacteriol.* (1993), 175(7), 2026-36) and the *UspA* universal stress gene is  
 35 has been shown to be inducible by osmotic stress (Alexander, et al., *Mol. Microbiol.* (1994), 11(6), 1059-71).

Although the literature teaches that stress genes are readily activated by osmotic stress, (a consequence of cell culture in small volumes), the present method has been adapted to use in very small volume assays. Screens have been

developed, utilizing the detector organism containing a bacterial stress promoter linked the LUX gene complex where the cells are suspended in about 1 ul to about 10 ul of media, where from about 2 ul to about 8 ul of media is preferred. The cells are stable in these conditions for about 60 to 120 min. without emitting a high baseline luminescence, indicating stress. The cells may be used in these volumes to detect a multiplicity of environmental insults.

#### Description of the Preferred Embodiments

The method of the present invention is designed to allow for the monitoring of samples for the presence of environmental insults by using a detector organism capable of demonstrating a change in bioluminescence in response to the presence of an insult. The transformed strains TV1060, TV1061, TV1076, WM1021, WM1202, WM1302 and WM1026 are all suitable and preferred for use as detector organisms. As with the preferred vectors, these detector organisms represent only a sample of the total number of detector organisms created for the purpose of detecting environmental insults. However, it will be readily apparent to one of skill in the art of molecular biology that the methods and materials used in their construction is representative of all other vectors described. Other preferred transformed detector organisms are listed in Table V of Example 10. At optimum growth conditions a baseline level of luminescence is produced by the detector organism. Introduction of an environmental insult to the actively growing cultures will induce the stress inducible promoter which will in turn activate the *lux* complex, resulting in an increase in the amount of light emitted from the detector organism. The amount of light emitted is correlated to the level of the insult. For the purpose of the present invention it is most preferred if the detector organisms are actively growing in log phase just prior to exposure to sample suspected of containing an insult and at a cell density of from between about 10 Klett Units to 50 Klett Units where about 20 Klett Units is most preferred. Light emission may be monitored by a variety of methods capable of detecting photons including but not limited to visual analysis, exposure to photographic film, scintillation counting or any device incorporating a photomultiplier tube where a luminometer similar to that produced by the DynaTech Corporation is most preferred.

In one embodiment varying concentrations of ethanol were used to apply stress to detector organism. As seen in Figure 4, a final concentration of 4% ethanol in the TV1060 cultures (containing the *grpE* promoter lux fusion) produced a dramatic increase in luminescence at 1000 seconds post-stress. Similarly in Figure 5, increasing concentrations of ethanol produced a corresponding increase in luminescence from the stressed cultures. Additionally, the organic pollutants, atrazine, pentachlorophenol (PCP), phenol, 2,4-dichloro-

phenoxy acetic acid (2,4-D), benzene, methanol, 2-nitrophenol, 4-nitrophenol, atrazine, toluene, dimethylsulfoxide, acetate, propionate, puromycin, 2,4-dichloroaniline, propanol, butanol, isopropanol, methylene chloride, Triton X100, acrylamide, methyl viologen, serine hydroxamate, menadione, ethidium bromide, mitomycin C and xylene as well as salts of the heavy metals copper, sulfate, lead nitrate, cadmium chloride, and mercury chloride copper were detected by the present method. Also detected were high osmotic strength, ultraviolet (U.V.) light irradiation, the oxidizing agents hydrogen peroxide and growth conditions of limiting phosphate, poor nitrogen source and poor carbon source. Chemicals were either dissolved in an appropriate solvent and added to cell cultures for testing or added directly to the growth media depending on their solubility properties. Benzene, ethanol, methanol, propanol, isopropanol, butanol, methylene chloride, dimethyl sulfoxide, Triton X-100, phenol, toluene, and xylene were added directly to LB medium, whereas 2-nitrophenol, 4-nitrophenol, and atrazine were first dissolved in methanol before being diluted into LB medium. Copper sulfate, lead nitrate, cadmium chloride, mercury chloride, sodium chloride, sodium acetate, sodium propionate, hydrogen peroxide, puromycin, methyl viologen, acrylamide, menadione, ethidium bromide, serine hydroxamate, and mitomycin C were first dissolved in water before being diluted into LB medium. Pentachlorophenol (PCP), 2,4-dichlorophenoxy acetic acid, and 2,4-dichloroaniline were first dissolved in ethanol and finally diluted into LB medium for testing. In all cases, the final concentration of either ethanol or methanol, or the slight dilution of the medium with water were such that it did not induce a significant response.

The data shown in Figure 7 and Tables II and III demonstrate two aspects of the present detection system. First, that organic and inorganic pollutants can be detected at low concentrations by the instant method and secondly, that the sensitivity of the system can be enhanced by the use of a host detector organism containing the *tolC*<sup>-</sup> mutation.

Figure 7 compares the relative sensitivities of detector organisms transformed with the GrpE.Lux.5 fusion with and without the *tolC*<sup>-</sup> mutation to the presence of pentachlorophenol. As can be seen by the data, the *tolC*<sup>-</sup> mutants exhibit significantly higher sensitivity to the presence of the PCP than the *tolC*<sup>+</sup> parental strain. Table III (Example 9) contains data comparing the relative sensitivities of detector organisms transformed with the pYR002 fusion with and without the *tolC*<sup>-</sup> mutation to the presence of PCP, 2,4-D, phenol, atrazine, ethanol, methanol, 2-nitrophenol and copper sulfate. Similarly, it is evident that PCP and 2,4-D are preferentially detected by the *tolC*<sup>-</sup> mutant host. The *tolC*<sup>-</sup> host also appears to be more sensitive to phenol, although to a lesser extent than with PCP and 2,4-D. The *tolC*<sup>-</sup> mutation appears to have little effect on the

sensitivity of the detector organism to non-organic contaminants such as copper sulfate which would be expected in light of the fact that the *tolC*<sup>-</sup> mutation is known to increase the host cell envelope permeability only to hydrophobic compounds.

Optionally, the method of the present invention may also be used to detect lethal levels of a insult by measuring the decrease in the baseline luminescence produced by the detector organism. Lethal levels of insults will interfere with either central metabolism or any *lux* protein function of the detector organism, which would be indicated by a decrease in light emitted from the cultures.

10      Figures 6a and 6b illustrate the sensitivity of the transformants WM1021 and WM1026 (containing the *dnaK* promoter lux fusion) to the stress of varying concentrations of ethanol. It is interesting to note that at the sublethal concentrations of ethanol varying from 1% to 4%, light emission increased in a fashion similar to the TV1060 cultures. By contrast, lethal concentrations of ethanol in the ranges of 8% to 16% produced a decrease in light emission from the detector cultures. Likewise, higher concentrations of PCP also result in decrease of light output in strain TV1076 (Figure 7). Thus, it is evident that the method of the present invention is capable of a bi-modular function. In one mode, detection of insults at sublethal levels are possible via the mechanism of the induction of the stress inducible promoter and the subsequent increase in light production from the detector organism. In an alternate mode, levels of insults capable of interfering with central cellular metabolism can also be detected since bioluminescence is an energy and reducing power dependant phenomenon and any interference with central metabolism will cause the baseline luminescence to decrease. Moreover, it is evident that the induction of light production from the stress promoter-lux fusions occurs at lower concentrations of specific pollutants than those concentrations required to result in a decrease of light output.

The following non-limiting Examples are meant to illustrate the invention but are not intended to limit it in any way.

## 30 EXAMPLES

## Materials and Methods

Restriction enzyme digestions, phosphorylations, ligations and transformations were done as described in Sambrook, J. et al., *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press. Isolation of restriction fragments from agarose gels used Qiagen columns (Qiagen, Inc.) and was performed as specified by the manufacturer. Strataclean (Stratagene, LaJolla, CA) and GeneClean (Bio101) were used to remove enzymes from restriction digests, as specified by the manufacturers. The meaning of

abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), and "d" means day(s).

#### EXAMPLE 1

##### Construction of plasmids pGrpELux.3 and pGrpELux.5 and transformation of RFM443

The outline of the scheme used to construct these plasmids is shown in Figure 1. Figure 1 is meant to illustrate the events of the construction, however DNA constructs are not drawn to scale.

Plasmid pGrpE4 was derived from pUC18 (Pharmacia, Cat. No. 27-4949-01) and contains the *Escherichia coli* stress gene, *grpE*, including its promoter sequences. pGrpE4 plasmid was digested with restriction enzymes EcoRI and BbuI and a 1.1 kb fragment was isolated following agarose gel electrophoresis corresponding to the *grpE* promoter and structural gene (Figure 1). The *grpE* promoter is conveniently bounded on the 5' end by an EcoRI site and on the 3' end by a PvuII site. The isolated fragment was further digested with restriction enzyme PvuII, separating the promoter region from the structural gene (Figure 1). An EcoRI linker fragment (Stragene, Catalog #901027) was phosphorylated and then ligated to the products of the PvuII digestion replacing the 3' PvuII site on the promoter with an EcoRI site. Further digestion with HaeIII produced a series of fragments, one of which contains the *grpE* promoter bounded on the 5' end by HaeIII and on the 3' end by EcoRI (Figure 1).

Plasmid pUCD615 (*J. Bacteriol.*, 169 (11) pp 5101-512, (1987)) is a 17.6 kb plasmid which contains the genes for kanamycin and ampicillin resistance and contains the promoterless *lux* gene operon with multiple cloning sites upstream of the start of *lux*. (Figure 1). The pUCD615 is first digested with restriction enzymes EcoRI and SmaI, opening the plasmid, followed by ligation with the DNA fragments from the HaeIII digestion (Figure 1).

In order to screen for active *grpE-lux* fusion ligated DNA was used to transform *E. coli* strain XL1Blue (Stratagene) by standard CaCl<sub>2</sub> transformation protocols and screened for the presence of plasmids using kanamycin resistance. Colonies were grown in gridded fashion on LB medium containing kanamycin (25 µg/mL) at 37 °C, overnight and were further screened for bioluminescence to determine which transformants contained promoter sequences fused to the *lux* genes of pUCD615. Bioluminescent screening was done by exposing the gridded colonies to X-OMAT AR film (Kodak, Rochester, NY) at ambient temperature and analyzing the developed films visually. Confirmation of transformation by the expected plasmid was further obtained by agarose gel electrophoretic analysis of plasmid DNA from cells producing light following restriction digests, for the presence and size of restriction fragments. Restriction fragments from Hind III,



BamHI, and Sal I digestions confirmed the presence and orientation of the *grpE* promoter in plasmids pGrpELux.3 and pGrpELux.5.

Plasmids pGrpELux.3 and pGrpELux.5 were moved by transformation into *E. coli* host strain RFM443 to give *E. coli* strains TV1060 and TV1061 respectively. *E. coli* RFM443 was originally derived from *E. coli* W3102 which is fully described in by B. Bachmann, in *E. coli* and *Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F. C. et al. Eds., pp 1190-1220, American Society of Microbiology, Washington, D.C. (1987))

#### EXAMPLE 2

##### Construction of Plasmid pRY006 and Transformation of RFM443

The outline of the scheme used to construct pRY006 is shown in Figure 2. Figure 2 is meant to illustrate the events of the construction, however DNA constructs are not drawn to scale.

DNA containing the *dnaK* promoter was obtained from Lambda phage 9E4 (Kohara, Y. et al; *Cell* 50, 495-508, 1987) using a Magic Lambda Prep, following the manufacturers suggested protocol (Promega Corp., Madison, WI). Phage DNA was digested with restriction enzymes BamHI and EcoRI. Several DNA fragments were liberated by this treatment including a 3.7 Kb BamHI-EcoRI restriction fragment encompassing the *dnaK* promoter region as described by Cowing et al (PNAS 82, 2679-2683, 1985), and approximately 3.0 kb of DNA 5' to this region, and 700bp 3' of the promoter region encoding the amino terminus of the DnaK protein. Digested DNA was ligated to pUCD615 which had been digested previously with restriction enzymes BamHI and EcoRI (Figure 2). The resulting plasmid constructs were used to transform *E. coli* strain DH5- $\alpha$  (GIBCO-BRL, Gaithersburg, MD, Catalog No. 82635a), and transformed bacteria were plated on LB medium containing 50  $\mu$ g/mL kanamycin, overnight at 37 °C. Resulting colonies were initially screened for bioluminescence by exposure to X-OMAT AR film as described in Example 1. Presence of the desired plasmid construction was confirmed by restriction enzyme analysis of plasmid DNA from transformed bacteria. Digestion with restriction enzymes BamHI and EcoRI yielded a 3.7 Kb restriction fragment following agarose gel electrophoresis, confirming the presence of the desired construction. This plasmid was designated pRY006. pRY006 was introduced into *E. coli* strain RFM443 by transformation creating strain WM1026.

EXAMPLE 3Construction of Plasmid pRY001 and pYR002  
and Transformation of RFM443

The outline of the scheme used to construct this plasmid is shown in

5 Figure 3. Figure 3 is meant to illustrate the events of the construction; however DNA constructs are not drawn to scale.

DNA containing the *dnaK* promoter (described by Cowing et al., PNAS 82, 2679-2683, 1985) was obtained from Lambda phage 9E4 (Kohara, Y. et al., Cell 50, 495-508, 1987) using a Magic Lambda Prep (Promega Corp., Madison, WI), following the protocol as described by the manufacturer. PCR amplification of the *dnaK* promoter region was accomplished using the following amplification primers:

Upper: 5'-GTTAGCGGATCCAAAAGCACAAAAAAT-3' (SEQ ID NO. 1)

Lower: 5'-AGCAGTGAATTCCATCTAAACGTCTCCA-3' (SEQ ID NO. 2)

15 DNA amplification was carried out as described by the manufacturer (GeneAmp PCR Reagent Kit, Perkin-Elmer Cetus, Norwalk, CT). Reagent concentrations were:

1X Buffer

200uM dATP

20 200uM dCTP

200uM dGTP

200uM dTTP

2.5 units Amplitaq Polymerase (Perkin-Elmer Cetus)

100pM Upper Primer

25 100pM Lower Primer

1ng 9E4 phage DNA Template

dH<sub>2</sub>O to 100 µL

The reaction was performed using a Perkin-Elmer Cetus GeneAmp PCR System 9600 thermal cycler programmed as follows:

30 Melting: 94 °C for 10 sec

Annealing: 50 °C for 10 sec

Extension: 72 °C for 15 sec

Cycles: 30

35 The amplified product which results is 207 base pairs in length, and contains the entire 182 bp segment encoding the *dnaK* promoter region as deposited in GeneBank (Accession 10420; Locus ECODNAK), as well as short 5' and 3' flanking sequences. PCR-amplified DNA was digested with restriction enzymes BamHI and EcoRI, and ligated to pUCD615 previously digested with restriction enzymes BamHI and EcoRI (Figure 3). The resulting plasmid

constructs were introduced into the *E. coli* strain DH5- $\alpha$  (GIBCO-BRL, Gaithersburg, MD, Cat. No. 82635a) by standard transformation protocols, and bacteria were plated on LB medium containing 50  $\mu$ g/mL kanamycin and grown overnight at 37 °C. Resulting colonies were initially screened for

5 bioluminescence by exposure to X-ray film as described in Example 1. Two colonies were picked for transformation analysis and the presence of the desired plasmid construction was confirmed by restriction enzyme analysis of plasmid DNA from transformed bacteria. Appearance of a 193 bp BamHI-EcoRI

10 restriction fragment following agarose gel electrophoresis confirmed the presence of the desired construction. These plasmids were designated pRY001 and pRY002. Although pRY001 and pRY002 represent different transformation colonies they are indistinguishable on the basis of restriction enzyme mapping and for the purposes of the present invention are considered identical. pRY001 and pRY002 were introduced into *E. coli* strain RFM443 by transformation creating

15 strains WM1021 and WM1022, respectively.

#### EXAMPLE 4

##### Stress induction of bioluminescence at 4% ethanol

Strain TV1060 was grown at 37 °C in LB medium containing kanamycin (25  $\mu$ g/mL) until it reached Klett 56 (measured on a Klett-Summerson colorimeter

20 with a #66 red filter) at which time it was diluted 1:11 into the same medium at ambient temperature and allowed to grow for 3 h at ambient temperature until reaching a density of 20 Klett Units. 100  $\mu$ L of cells were placed into the wells of a microtiter plate followed by the addition of either 10  $\mu$ L 40% ethanol (experimental, final concentration 4% ethanol) or 10  $\mu$ L of distilled water

25 (control). The plate was immediately placed into a luminometer (Luminoskan, Finland) and bioluminescence was measured as RLU vs. time. As can be seen in Figure 4, light emission from the transformed TV1060 cultures stressed with 4% ethanol increased dramatically 1000 sec post-stress reaching a maximum level of 130 RLU. Addition of the distilled water to the control cultures produced no

30 variation in the baseline luminescence (Figure 4).

#### EXAMPLE 5

##### Stress induction of bioluminescence at varying concentrations of ethanol

Strain TV1060 was grown overnight in LB medium containing kanamycin

35 (25  $\mu$ g/mL) and then diluted 1:100 in the same medium and grown at room temperature until reaching a Klett of 20. 100  $\mu$ L of cells were placed into the wells of a microtiter plate containing 100  $\mu$ L of either 2%, 4%, 8% or 16% (giving final concentrations of 1%, 2%, 4%, and 8% respectively of ethanol, experimental) or 100  $\mu$ L of the same medium (control). The plate was

immediately placed into a luminometer, model ML3000 (Dynatech Laboratories, Chantilly, VA) and bioluminescence was measured as RLU vs. time. As can be seen in Figure 5, light emission from the transformed TV1060 cultures stressed with ethanol demonstrated an increase in luminescence corresponding to increasing concentrations of ethanol. As in Example 4, increases in luminescence were observed after 1000 sec post-stress. Control cultures produced no variation in the baseline luminescence (Figure 5). Maximum light emission was obtained with 8% ethanol at 3000 sec post-stress showing a 263 fold increase in light production over the control. Lower levels of ethanol stress produced correspondingly lower levels of light output where 4%, 2%, and 1% ethanol concentrations gave 96, 13.5 and 3.9 fold increases in light emission over the controls, respectively.

#### EXAMPLE 6

##### Stress Induction of Bioluminescence In

##### Strains WM1021 and WM1026

Strains WM1021 and WM1026 were grown at 37 °C for approximately 18 h in LB medium containing kanamycin (50 µg/mL). Cultures were then diluted 1:50 into the fresh media, and grown at ambient temperature for approximately 3 h. When cells reached a density of Klett Units of 20, 80 µL of the cell suspension was placed into the wells of a microtiter plate containing 20 µL of ethanol at various concentrations, and the plate was immediately placed into a Dynatech Model ML3000 luminometer. Ethanol was present at final concentrations of 16%, 8%, 4%, 2%, and 1%, and a deionized water control was included. The luminometer was previously programed to measure luminescence at 2 min intervals. Figures 6a and 6b present the resulting luminescence data produced in response to varying concentrations of ethanol by strains WM1021 and WM1026, respectively. As shown in Figure 6a luminescence increases sharply 1000 sec post-stress in those cultures receiving the sublethal concentrations of 1%, 2% and 4% ethanol. At the higher, lethal concentrations of ethanol of 8% and 16%, luminescence was seen to decline below baseline values, suggesting cell death. Figure 6b shows similar results using the host cell WM1026. Thus, light is increased in response to sublethal levels of ethanol whereas light output is diminished at higher, lethal levels. This example demonstrates the ability of the invention to detect the presence of both sublethal and lethal levels of an insult.

### EXAMPLE 7

#### Comparison of stress induction of bioluminescence by pentachlorophenol in *tolC*<sup>+</sup> and *tolC*<sup>-</sup> strains transformed with pGrpE.Lux.5

5 In order to provide an *E. coli* detector organism with enhanced permeability to organic compounds the *tolC*<sup>-</sup> mutant DE112 was constructed from RFM443. The *E. coli* strain DE112 is isogenic to strain RFM443 except for the mutation at the *tolC* locus. It was constructed by phage P1 mediated transduction using a lysate grown on strain CS1562 (*tolC*::miniTn10) as a donor and strain  
10 RFM443 as a recipient. CS1562 (*tolC*::miniTn10) is fully described in Austin et al., *J. Bacteriol.* 172, 5312, (1990) and the transduction procedure is described in Miller, J. H., *Experiments in Molecular Genetics*, (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 201-205. RFM443 was originally derived from W3102 which is fully described in by B. Bachmann, in *E. coli and*  
15 *Salmonella typhimurium: Cellular and Molecular Biology* (Niedhardt et al. Eds., pp 1190-1220, American Society of Microbiology, Washington DC, (1987)). Resultant tetracycline resistant recombinants were screened for hypersensitivity to the hydrophobic compound crystal violet.

DE112 and RFM443 are isogenic except at the *tolC* locus. Both strains  
20 were transformed with the plasmid pGrpELux.5 as described in Example 1, and designated TV1076 (*tolC*::miniTn10) and TV1061 (*tolC*<sup>+</sup>) respectively.

Both strains were grown overnight at 25 °C in LB medium containing Kanamycin at 50 µg/mL. The overnight cultures were diluted 1:50 into fresh LB medium containing Kanamycin (50 µg/mL) and were incubated with shaking at  
25 25 °C for 4 h. Cell density was determined on a Klett-Summerson colorimeter. When both strains reached readings of between 25 and 29 Klett Units the cells were used for testing.

Cells (50 µL) from each culture were added to LB medium containing Kanamycin (50 µg/mL) and various concentrations of pentachlorophenol (PCP) in  
30 the wells of a microtiter plate such that the final volume in each well was 100 µL.

A 100 mg/mL stock solution of PCP in ethanol was used to make a 75 µg/mL solution of PCP in LB medium containing Kanamycin (50 µg/mL). This solution (50 µL) was placed in wells of a microtiter plate and serial two-fold dilutions were made of from these. When the equal volume of cells was added,  
35 the highest ethanol concentration was 0.037%, a concentration known not to induce a significant response from these cells.

Light readings were taken at periodic intervals, in a Dynatech ML3000 microtiter plate luminometer at 25 °C, and the data is shown in the graph of

Figure 7 plotted as concentration of PCP as a function of induction ratio. The induction ratio is defined as the light output (RLU) in the presence of PCP divided by the light output (RLU) in the absence of PCP.

Figure 7 shows the induction ratio at 60 min after the addition vs. the dose of PCP. At low doses, no induction of light output (ratio = 1) from the *tolC*<sup>+</sup> strain, TV1061, was seen. However, at these lower doses the *tolC*<sup>-</sup> strain, TV1076, showed significant increase of light output. Also, at higher doses, the induction in the *tolC*<sup>+</sup> strain is observed, and a toxic effect of the PCP (loss of light output) is observed in the *tolC*<sup>-</sup> strain. Thus, the *tolC*<sup>-</sup> strain is useful for detecting lower concentration of this compound than can be detected in the *tolC*<sup>+</sup> strain. Likely, other hydrophobic compounds will also be more readily detected by the combination of this host strain with the plasmids containing *grpE::lux* fusions

#### EXAMPLE 8

Stress induction of bioluminescence by organic and inorganic pollutants in *tolC*<sup>+</sup> and *tolC*<sup>-</sup> strains transformed with pGrpE.Lux.5

TV1061 and TV1076 were engineered as described above. Both strains were grown overnight at 25 °C in LB medium containing Kanamycin at 50 µg/mL. The overnight cultures were diluted between 40 and 100-fold into fresh LB medium containing Kanamycin (50 µg/mL) and were incubated with shaking at 25 °C. Cell density was determined on a Klett-Summerson colorimeter. When both strains gave readings of between 15 and 30 Klett Units, cells were used for testing.

Cells (50 µL) from each culture were added to LB medium containing Kanamycin (50 µg/mL) and various concentrations of benzene, ethanol, methanol, 2-nitrophenol, 4-nitrophenol, PCP, phenol, toluene, and xylene in the wells of a microtiter plate such that the final volume in each well was 100 µL. Control wells contained appropriate volumes of solvents used to dissolve the test compounds.

Benzene, ethanol, methanol, propanol, isopropanol, butanol, methylene chloride, dimethyl sulfoxide, Triton X-100, phenol, toluene, and xylene were added directly to LB medium containing kanamycin (50 µg/mL). Stock solutions in methanol of 2-nitrophenol (136 mg/mL) and of 4-nitrophenol (112 mg/mL) were diluted into LB medium containing kanamycin (50 µg/mL) to concentrations tested. The final concentration of methanol was such that it did not induce a significant response from these cells. A stock solution in ethanol of PCP (100 mg/mL) was diluted into LB medium containing kanamycin (50 µg/mL) to the concentration tested. The final concentration of ethanol was such that it did

not induce a significant response from these cells. Stock solutions in water of copper sulfate (250 mM), lead nitrate (100 mM), mercury chloride (100 mM), sodium chloride (20%), sodium acetate (2 M), sodium propionate (2 M), hydrogen peroxide (30%), puromycin (10 mg/mL), methyl viologen (200 mg/mL), and acrylamide (1 M) were diluted into LB medium containing kanamycin (50 µg/mL) to the concentrations tested. A stock solution in water of cadmium chloride (100 mM) was diluted into LB medium lacking kanamycin to the concentrations tested.

Light readings were taken at periodic intervals over a two h period and the data is shown in Table II. All luminescence readings were measured in a Dynatech ML3000 microtiter plate luminometer at 25 °C.

Data in Table II represent readings taken 1 h post exposure to the test compounds and are expressed as the concentration of test compound that gives the maximum luminescence. Data are also given showing the fold increase in induced luminescence over the baseline luminescence.

**TABLE II**  
Induction of Light Production from  
Plasmid pGrpELux.5 by Chemicals

<u>Chemical</u>	<u>TV1061 (<i>tolC</i><sup>+</sup>)</u>		<u>TV1076 (<i>tolC</i><sup>-</sup>)</u>	
	<u>Conc. for</u> <u>Max Induction</u>	<u>Fold</u> <u>Increase</u>	<u>Conc. for</u> <u>Max Induction</u>	<u>Fold</u> <u>Increase</u>
Benzene	10 mg/mL*	1.8	10 mg/mL*	2
Ethanol	2% (v/v)	182	2% (v/v)	140
Methanol	4% (v/v)	40	4% (v/v)	17
2-nitrophenol	113 µg/mL	2.5	113 µg/mL	2.5
4-nitrophenol	37 µg/mL	4.7	37 µg/mL	3
Pentachlorophenol	37.5 µg/mL*	15.3	1.17 µg/mL	6.7
Phenol	460 µg/mL	12	460 µg/mL	11.1
Toluene	10 mg/mL*	2.7	10 mg/mL*	3.2
Xylene	3.3 mg/mL	1.7	3.3 mg/mL	1.4
2,4-Dichlorophenoxy acetic acid	133 µg/mL	13.1	133 µg/mL	9.3
Copper Sulfate	1000 µg/mL	6.3	1000 µg/mL	5.5
Dimethyl Sulfoxide	1% (v/v)*	2.9	1% (v/v)	2.6

**TABLE II**  
Induction of Light Production from  
Plasmid pGrpELux.5 by Chemicals

<u>Chemical</u>	<u>TV1061 (<i>tolC</i><sup>+</sup>)</u>		<u>TV1076 (<i>tolC</i><sup>-</sup>)</u>	
	<u>Conc. for</u> <u>Max Induction</u>	<u>Fold</u> <u>Increase</u>	<u>Conc. for</u> <u>Max Induction</u>	<u>Fold</u> <u>Increase</u>
Lead	800 $\mu$ M*	2.4		
Sodium Chloride	2 % (w/v)*	9.2		
Cadmium Chloride	333 $\mu$ M	9.7		
Sodium Acetate	50 mM	14		
Sodium Propionate	100 mM*	76		
Hydrogen Peroxide	0.0093% (w/v)	54.5	0.0046% (w/v)	6.1
Puromycin	50 $\mu$ g/mL	1.6	Only inhibition detected	
Mercury Chloride	0.39 $\mu$ M	1.4	0.39 $\mu$ M	1.6
2,4-dichloroaniline	100 $\mu$ g/mL*	49.4	100 $\mu$ g/mL*	49.4
Propanol	2 % (v/v)	84.8		
Isopropanol	2% (v/v)	124		
Butanol	0.5% (v/v)	27.7		
Methylene Chloride	4% (v/v)	11.4		
Triton X-100	1% (v/v)*	1.2		
Methyl Viologen	50 $\mu$ g/mL	2		
Acrylamide	50 mM*	1.9		

\*Maximum concentration tested

It is evident from the data in Table II that a wide variety of chemical compounds and environmental conditions will induce bioluminescence from *E. coli* strains containing the plasmid pGrpELux.5. Hence, it may be concluded that detector organisms containing the *tolC*<sup>-</sup> mutation represent a preferred host for some environmental insults.

#### EXAMPLE 9

##### Stress induction of bioluminescence by organic and inorganic pollutants in *tolC*<sup>+</sup> and *tolC*<sup>-</sup> strains

##### transformed with pRY002

DE112 (*tolC*::miniTn10) was engineered as described in Example 7, and the source of RFM443 has been previously discussed. Both strains were transformed with the plasmid pRY002 by standard methods as described in Example 3, and designated WM1302 (*tolC*::miniTn10) and WM1202 (*tolC*<sup>+</sup>) respectively.



Both strains were grown overnight at 25 °C in LB medium containing Kanamycin at 50 µg/mL. The overnight cultures were diluted 1:100 into fresh LB medium containing Kanamycin (50 µg/mL) and were incubated with shaking at 25 °C for 4 h. Cell density was determined on a Klett-Summerson colorimeter.

5 When both strains gave readings of 28 Klett Units, cells were used for testing.

Cells (50 µL) from each culture were added to wells of a microtiter plate containing LB medium containing Kanamycin (50 µg/mL) and various concentrations of PCP, 2,4-dichlorophenoxyacetic acid (2,4-D), phenol, ethanol, methanol, 2-nitrophenol, atrazine and copper sulfate such that the final volume in  
10 each well was 100 µL. Control wells contained appropriate volumes of solvents used to dissolve the test compounds.

Chemicals were prepared for testing essentially as described in Example 8. Copper sulfate was added directly to the culture medium and atrazine was first dissolved in methanol before additions to cell cultures. With the exception of  
15 atrazine, all solvents were added at levels below those needed to see a stress response in the system. The level of methanol present in the atrazine sample induced a low but detectable response; data in Table III is the net response detected above the response obtained with an equivalent amount of methanol alone.

20 Light readings were taken at periodic intervals over a two h period and the data is shown in Table III. All luminescence readings were measured in a Dynatech ML3000 microtiter plate luminometer at 25 °C.

Data in Table III represent readings taken 1 h post exposure to the test compounds and are expressed as the concentration of test compound that gives the  
25 maximum luminescence. Data are also given showing the fold increase in induced luminescence over the baseline bioluminescence.

**TABLE III**  
Induction of Bioluminescence from Cells  
Transformed with Plasmid pRY002 by Chemicals

<u>Chemical</u>	<u>WM1202 (<i>tolC</i><sup>+</sup>)</u>		<u>WM1302 (<i>tolC</i><sup>-</sup>)</u>	
	<u>Conc. for</u> <u>Max Ind.**</u>	<u>Fold</u> <u>Incr.***</u>	<u>Conc. for</u> <u>Max. Ind.</u>	<u>Fold</u> <u>Incr.</u>
Atrazine	135 µg/mL*	2.2	no ind.	-
Copper Sulfate	3.3mM*	9	3.3 mM*	8.4
Ethanol	4% (v/v)	1264	4% (v/v)	1525
2,4-D	400 µg/mL*	42	100 µg/mL	140
Methanol	5% (v/v)	3.7	5% (v/v)	3.9
2-nitrophenol	340 µg/mL*	7.6	340 µg/mL*	9.3
PCP	37.5 µg/mL*	106	1.2 µg/mL	45
Phenol	1.4 mg/mL	6	1.4 mg/mL	72

\*Maximum concentration tested

\*\*"Conc. for Max. Ind." means concentration for maximum induction

\*\*\*"Fold Incr." means the fold increase over baseline bioluminescence

It is evident from the data in Table III that several classes of chemical compounds will induce bioluminescence from *E. coli* strains containing the plasmid pRY002. Both the *tolC*<sup>-</sup> and *tolC*<sup>+</sup> hosts demonstrated similar sensitivities to less hydrophobic compounds. It may be concluded that detector organisms containing the *tolC*<sup>-</sup> mutation represent a preferred host for some environmental insults.

#### EXAMPLE 10

Construction of promoters *lon*, *recA*, *uvrA*, *katG*,  
*micF*, *uspA*, *xthA*, *his*, *lac*, *phoA*, *glnA*  
fused to the *lux* operon

10

15

The scheme to construct additional plasmids is identical to the construction of pRY001 and pRY002 as illustrated in Figure 3 and described in Example 3, with the exception that different primers and templates were used for the PCR reactions, and that 40 cycles were used in PCR reactions. The primers and templates used are listed below in Table IV.

TABLE IV

Target Promoter <sup>a</sup>	Upper Primer (5' to 3') <sup>b</sup>	Lower Primer (5' to 3') <sup>c</sup>	Template <sup>d</sup>	Product Size (bp) <sup>e</sup>
<i>lon</i>	ACTTAAGGATCCAAAGCGATGGCGCGTAAAA	AGCAGCGAATTCATCGCCGCTTCCAGACAA	148	534
<i>recA</i>	ACTTAAGGATCCAGAGAAAGCCTGTCGGCAC	AGCTTTGAATTCGCTTCTGTTGTTTT	c	279
<i>uvrA</i>	ACTTTGGATCCGCTGTAAACGCGGATTG	AGCAGCGAATTCCTCCCGGATTAAACGCTT	637, 638	225
<i>kaiG</i>	ACTTAAGGATCCCGAAATGAGGGCGGGAAA	AGCAGCGAATTCGAACGTTGCTGACCACGA	538	654
<i>micF</i>	ACTTAAGGATCCCGCCCAAAATGCAGAATA	AGCAGCGAATTCGGGCATCCGGTTGAAATAG	373	331
<i>fabA</i>	ACTTAAGGATCCCGCCATTACGTTGGCTGAA	AGCAGCGAATTCGCCACCCGTTTCGGTCAAT	222	260
<i>uspA</i>	ACTTAAGGATCCCTCCCGATAGCGTGCCA	AGCAGCGAATTCGGCGATGAGAAATGTGTTAT	608	381
<i>xthA</i>	ACTTAAGGATCCAAATTACTGCGCCATTCTG	ACATCGGAATTCATCATAGTCGCTGCCATTT	c	181
<i>his</i>	ACTTAAGGATCCCTAATTGTACGCATGTCA	AGCAGCGAATTCAAAAGTCTCTGTGAATGTT	350	301
<i>phoA</i>	ACTTAAGGATCCAGATTATCGTCACTGCAA	AGCAGCGAATTCGGCCCAATCAGCAAAATAA	141, 142	501
<i>glnA</i>	ACTTTCGGATCCCTTGGTGCAACATTACAT	AGCAGCGAATTCCTCAGCGGACATCGTCAGT	546	227

<sup>a</sup>Promoter sequences are available in Genbank.

<sup>b</sup>The underlined region of the upper primer is a *Bam*HI restriction enzyme cleavage site. 3' to the restriction site is sequence (generally 18 nucleotides) complementary to region upstream of the desired promoter.

<sup>c</sup>The underlined region of the lower primer is an *Eco*RI restriction enzyme cleavage site. 3' to the restriction site is sequence (generally 18 nucleotides) complementary to the region downstream of the desired promoter.

<sup>d</sup>The template for PCR reactions was either a chromosomal DNA preparation (C, prepared according Zyskind & Bernstein, Recombinant DNA Laboratory Manual, Academic Press, New York, 1992) or a water-resuspended plaque from one of an overlapping set of specialized  $\lambda$  transducing phages covering the *E. coli* chromosome [Kohara et al. Cell 50: 495-508 (1987)] indicated by its assigned number [see Bouffard et al., CABIOS 8:563-657 (1992)] grown on *E. coli* K12. Amplification from plaques was performed by the method of Berg [Kirschner, Blakesley and Berg (1992) Nucleic Acids Research 19:1153] while that from the chromosomal preparation differed from that of Berg that 1  $\mu$ l of the chromosomal DNA preparation was used as template.

<sup>e</sup>The calculated size of the PCR product. Agarose gel electrophoretic analysis confirmed product size.

All of the promoter sequences listed in Table IV are published and available from GenBank. The underlined region of the upper primer is a *Bam*HI restriction enzyme cleavage site. 3' to the restriction site is sequence (generally 18 nucleotides) complimentary to the region upstream of the desired promoter. The underlined region of the lower primer is an *Eco*RI restriction enzyme cleavage site. 3' to the restriction site is sequence (generally 18 nucleotides) complementary to the region downstream of the desired promoter. The template for PCR reactions was either a chromosomal DNA preparation (C. prepared according to Zyskind & Bernstein, Recombinant DNA Laboratory Manual, Academic Press, New York, 1992) or a water resuspended plaque from one of an overlapping set of specialized  $\lambda$  transducing phages covering the *E. coli* chromosome (Kohara et al., *Cell* 50; 495-508 (1987)) indicated by its assigned number (Bouffard et al., *CABIOS* 8: 563-567 (1992)) grown on *E. coli* K12. Amplification from plaques was performed by the method of Berg et al., *Nucleic Acids Research* 19,1153, (1991) Amplifications from the chromosomal preparations differed from that of Berg in that 1  $\mu$ L of the chromosomal DNA preparation was used as template. The calculated size of the PCR product was confirmed by agarose gel electrophoresis.

Each of the resultant plasmids were placed in three *E. coli* host strains: RFM443, DE112, and W3110. Plasmids and transformed host cells are listed below in Table V.

TABLE V

<u>Promoter</u>	<u>Plasmids</u>	<u>E. coli host</u>	<u>Strain name</u>
<i>lon</i>	pLonE6	W3110	pLonE6/W3110
	pLonE6	RFM443	pLonE6/RFM443
	pLonE6	DE112	pLonE6/DE112
	pLonF1	W3110	pLonF1/W3110
	pLonF1	RFM443	pLonF1/RFM443
	pLonF1	DE112	pLonF1/DE112
<i>recA</i>	pRecALux1	W3110	DPD2789
	pRecALux2	W3110	DPD2790
	pRecALux3	W3110	DPD2791
	pRecALux1	RFM443	DPD2792
	pRecALux2	RFM443	DPD2793
	pRecALux3	RFM443	DPD2794

<u>Promoter</u>	<u>Plasmids</u>	<u>E. coli host</u>	<u>Strain name</u>
<i>uvrA</i>	pRecALux1	DE112	DPD2795
	pRecALux2	DE112	DPD2796
	pRecALux3	DE112	DPD2797
	pUvrALux1	W3110	DPD2814
	pUvrALux2	W3110	DPD2815
	pUvrALux3	W3110	DPD2816
	pUvrALux4	W3110	DPD2817
	pUvrALux1	RFM443	DPD2818
	pUvrALux2	RFM443	DPD2819
	pUvrALux3	RFM443	DPD2820
	pUvrALux4	RFM443	DPD2821
	pUvrALux1	DE112	DPD2822
<i>katG</i>	pKatGLux2	W3110	DPD2507
	pKatGLux6	W3110	DPD2508
	pKatGLux2	DE112	DPD2509
	pKatGLux6	DE112	DPD2510
	pKatGLux2	RFM443	DPD2511
	pKatGLux6	RFM443	DPD2512
<i>micF</i>	pMicFLux1	W3110	DPD2515
	pMicFLux2	W3110	DPD2516
	pMicFLux1	DE112	DPD2517
	pMicFLux2	DE112	DPD2518
	pMicFLux1	RFM443	DPD2519
	pMicFLux2	RFM443	DPD2520
<i>uspA</i>	pUspALux.2	W3110	DE130
	pUspALux.2	RFM443	DE134
	pUspALux.2	DE112	DE138

<u>Promoter</u>	<u>Plasmids</u>	<u>E. coli host</u>	<u>Strain name</u>
	pUspALux.6	W3110	DE142
	pUspALux.6	RFM443	DE146
	pUspALux.6	DE112	DE150
	pUspALux.13	W3110	DE154
	pUspALux.13	RFM443	DE158
	pUspALux.13	DE112	DE162
<i>xthA</i>	pXthALux1	W3110	DPD2771
	pXthALux2	W3110	DPD2772
	pXthALux3	W3110	DPD2773
	pXthALux4	W3110	DPD2774
	pXthALux5	W3110	DPD2775
	pXthALux6	W3110	DPD2776
	pXthALux1	RFM443	DPD2777
	pXthALux2	RFM443	DPD2778
	pXthALux3	RFM443	DPD2779
	pXthALux4	RFM443	DPD2780
	pXthALux5	RFM443	DPD2781
	pXthALux6	RFM443	DPD2782
	pXthALux1	DE112	DPD2783
	pXthALux2	DE112	DPD2784
	pXthALux3	DE112	DPD2785
	pXthALux4	DE112	DPD2786
	pXthALux5	DE112	DPD2787
	pXthALux6	DE112	DPD2788
<i>his</i>	pHisLux5	RFM443	DPD1534
	pHisLux9	RFM443	DPD1535
	pHisLux12	RFM443	DPD1536
	pHisLux5	W3110	DPD1537
	pHisLux9	W3110	DPD1538
	pHisLux12	W3110	DPD1539
	pHisLux5	DE112	DPD1540
	pHisLux9	DE112	DPD1541
	pHisLux12	DE112	DPD1542

<u>Promoter</u>	<u>Plasmids</u>	<u>E. coli host</u>	<u>Strain name</u>
<i>lac</i>	pLacLux	W3110	TVI063
	pLacLux	RFM443	TVI058
	pLacLux	RFM443	TVI068
	pLacLux	DE112	TVI073
<i>phoA</i>	pPhoALux3	W3110	DPD1522
	pPhoALux4	W3110	DPD1523
	pPhoALux5	W3110	DPD1524
	pPhoALux11	W3110	DPD1525
	pPhoALux3	RFM443	DPD1526
	pPhoALux4	RFM443	DPD1527
	pPhoALux5	RFM443	DPD1528
	pPhoALux11	RFM443	DPD1529
	pPhoALux3	DE112	DPD1530
	pPhoALux4	DE112	DPD1531
	pPhoALux5	DE112	DPD1532
	pPhoALux11	DE112	DPD1533
<i>glnA</i>	pGlnALux1	W3110	DPD2830
	pGlnALux2	W3110	DPD2831
	pGlnALux3	W3110	DPD2832
	pGlnALux4	W3110	DPD2833
	pGlnALux1	RFM443	DPD2834
	pGlnALux2	RFM443	DPD2835
	pGlnALux3	RFM443	DPD2836
	pGlnALux4	RFM443	DPD2837
	pGlnALux1	DE112	DPD2838
	pGlnALux2	DE112	DPD2839
	pGlnALux3	DE112	DPD2840
	pGlnALux4	DE112	DPD2841

Promoter-reporter fusions were tested in transformed detector host cells using a variety of environmental insults, appropriate to the known sensitivity of the promoter. Promoters and their corresponding inducing insults are summarized in Table VI.

TABLE VI

<u>Target Promoter</u>	<u>Upset<sup>b</sup></u>	<u>Observed Induction by<sup>c</sup></u>	<u>Expression lacking in<sup>d</sup></u>
<i>lon</i>	protein damage	ethanol <sup>e</sup> , copper sulfate	
<i>recA</i>	DNA damage	mitomycin C <sup>f</sup> , cadmium chloride <sup>g</sup> , U.V. light ethidium bromide	
<i>uvrA</i>	DNA damage	U.V. light	
<i>katG</i>	oxidative damage	methyl viologen <sup>h</sup> , hydrogen peroxide <sup>i</sup> , menadione <sup>h</sup>	
<i>micF</i>	oxidative damage	methyl viologen, hydrogen peroxide	
<i>uspA</i>	any	ethanol <sup>j</sup> , copper sulfate	
<i>xthA</i>	stationary phase	acetate, propionate, hydrogen peroxide	
<i>his</i>	amino acid starvation	serine hydroxamate <sup>k</sup>	<i>relA spoT</i>
<i>lac</i>	carbon starvation	absence of glucose as a C source <sup>l</sup>	
<i>phoA</i>	phosphate limitation	low phosphate levels <sup>m</sup>	
<i>glnA</i>	nitrogen limitation	glutamine as sole N source <sup>n</sup>	

<sup>b</sup> causing a pleiotropic regulatory response.

<sup>c</sup> chemical induction turning on an increased bioluminescent response.

<sup>d</sup> genetic construction preventing bioluminescent expression by disruption of a positive regulatory circuit.

<sup>e</sup> ethanol is a strong inducer of the heat shock response [Neidhardt and VanBogelen in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1334-1345, American Society of Microbiology, Washington, DC (1987))]

<sup>f</sup> mitomycin C is a known inducer of the SOS response [Walker in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1346-1357, American Society of Microbiology, Washington, DC (1987))].

<sup>g</sup> cadmium has been reported to induce genetic damage [Neidhardt and VanBogelen in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1334-1345, American Society of Microbiology, Washington, DC (1987))].

<sup>h</sup> this compound promotes redox cycling producing superoxide. Superoxide is dismutated to hydrogen peroxide. Superoxide induces synthesis of 40 proteins in addition to the 40 proteins induced by exposure to hydrogen peroxide [Dempsey, *Ann. Rev. Genet.* 25: 315-337 (1991)].

<sup>i</sup> hydrogen peroxide induces the *oxyR* regulon and several other proteins among the approximately 40 polypeptides induced in *E. coli* and *S. typhimurium* [Christman et al. *Cell* 41: 753-762 (1985); Storz et al. *Science* 248: 189-194 (1990); Dempsey, *Ann. Rev. Genet.* 25: 315-337 (1991)].



- j T. Van Dyk. unpublished.
- k this chemical prevents aminoacylation of tRNA<sup>Ser</sup> thus inducing the stringent response [Cashel and Rudd in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1410-1438, American Society of Microbiology, Washington, DC (1987); Winkler in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 395-411, American Society of Microbiology, Washington, DC (1987)].
- l the catabolite activation response is triggered by the absence of a good carbon source [Neidhardt, Ingraham and Schaecter. Physiology of the Bacterial Cell: A Molecular Approach, Sinauer Associates, Sunderland, MA (1990), pp. 351-388; Magasanik and Neidhardt in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1318-1325, American Society of Microbiology, Washington, DC (1987)].
- m use of limiting phosphate concentrations induces the phosphate starvation regulon [Wanner in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1326-1333, American Society of Microbiology, Washington, DC (1987)].
- n use of glutamine as a sole N source induces expression of the N starvation regulon [Rietzer and Magasanik in *E. coli and Salmonella typhimurium*; Cellular and Molecular Biology (Neidhardt, F.C. et al. Eds., pp. 1302-1320, American Society of Microbiology, Washington, DC (1987)].

### EXAMPLE 11

#### Response of *lon* transformed host cell to

#### ethanol or copper sulfate

- 5 *E. coli* strain pLonE6/RFM443 was grown overnight at 26 °C in LB medium containing Kanamycin (50 µg/mL) and diluted into the fresh LB medium containing Kanamycin (50 µg/mL) and grown at 26 °C to early log-phase. 50 µL of cells were added to 50 µL of LB medium containing Kanamycin (50 µg/mL) and various concentrations of ethanol (added directly to the medium) or copper
- 10 sulfate (diluted from a 250 mM stock solution in water). Light output was quantitated as a function of incubation time in a Dynatech ML3000 luminometer at 26 °C. The maximum response induced by ethanol was observed when the final concentration of ethanol was 4%; at 60 min after addition of ethanol the luminescence was 134 fold greater in the presence of ethanol than in the untreated
- 15 control. The maximum copper sulfate induction resulted when the final concentration was 5 mM; at 60 min the induction ratio was 408 fold.

### EXAMPLE 12

#### Response of *recA* transformed host cell to

#### mitomycin C, ethidium bromide or cadmium chloride

- 20 *E. coli* strains containing plasmid pRecALux3 were grown overnight at 26 °C in LB medium containing Kanamycin (50 µg/mL) and diluted into the fresh LB medium containing Kanamycin (50 µg/mL) and grown at 26 °C to early log-phase. 50 µL of cells were added to 50 µL of LB medium containing Kanamycin

(50 µg/mL) and various concentrations of mitomycin C (diluted from a 2 mg/ml stock solution in water). Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C. At 100 min after addition of 0.5 µg/mL mitomycin C, the induction ratios were as follows:

5

Strain DPD2791	(pRecALux3/W3110)	4.74
Strain DPD2794	(pRecALux3/RFM443)	20.00
Strain DPD2797	(pRecALux3/DE112)	15.70

- 10 *E. coli* strain DPD2794 demonstrated response to the presence of ethidium bromide. Cells were grown overnight at 26 °C in LB medium containing kanamycin (25 µg/mL) and diluted into the fresh LB medium and grown at 26 °C to early log-phase. 50 µL of cells were added to 50 µL of LB medium containing various concentrations of ethidium bromide (diluted from a 10 mg/mL stock solution in water). Light output was quantitated in a Dynatech ML3000
- 15 luminometer at 26 °C. At 180 min after addition of 0.25 mg/mL ethidium bromide, the induction ratio was 1.9 fold.

*E. coli* strain DPD2794 was also shown to respond to the presence of cadmium chloride by a disk diffusion assay. Cells were spread on an LB agar plate containing Kanamycin (50 µg/mL) and a filter disk which had been wet with 20 µl of a 100 mM cadmium chloride solution was placed on the agar plate. Following incubation overnight at 37 °C, the agar plate was allowed to cool to room temperature. DuPont Reflection™ film was exposed to the plate for 10 min. Surrounding a zone of growth inhibition (18 mm diameter) a zone of enhanced

25 bioluminescence (35 mm diameter) was observed.

### EXAMPLE 13

#### Response of *KatG* transformed host cell to methyl viologen, hydrogen peroxide or menadione

- E. coli* strains containing plasmids pKatGLux2 and pKatGLux6 were
- 30 grown overnight at 37 °C in LB medium and diluted into fresh LB medium and grown at 37 °C to early log-phase. 40 µL of cells were added to 60 µL of LB medium and various concentrations of methyl viologen (MV) which was diluted from a 200 mg/mL stock solution in water or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) which was diluted from a 0.3% solution in water. Light output was quantitated in a
- 35 Dynatech ML3000 luminometer at 26 °C. Data is shown below in Tables VII and VIII.

TABLE VII

Strain	Time of Max Ind.	[MV] for Max Ind.	Induction Ratio
DPD2507	60 min	8.75 mM*	56.7
DPD2508	60 min	8.75 mM*	19.5
DPD2509	60 min	8.75 mM*	31.9
DPD2510	80 min	8.75 mM*	11.2
DPD2511	60 min	8.75 mM*	226
DPD2512	50 min	2.2 mM	50.7

\*Maximum concentration tested.

TABLE VIII

Strain	Time of Max Ind.	[H <sub>2</sub> O <sub>2</sub> ] for Max Ind.	Induction Ratio
DPD2507	65 min	1.65 mM	70.5
DPD2508	65 min	1.65 mM	3034
DPD2509	55 min	0.41 mM	250
DPD2510	50 min	0.41 mM	810
DPD2511	55 min	0.41 mM	376
DPD2512	45 min	0.41 mM	6067

*E. coli* strain DPD2511 was also shown to respond with increased bioluminescence to the presence of menadione. Cells were grown overnight in LB medium containing kanamycin (25 µg/mL) at 26 °C and diluted to LB medium and grown to log-phase at 26 °C. 20 µL of cells were added to wells of microtiter plates containing various concentrations of menadione (diluted from a 200 mg/mL solution in water) in 80 µL of LB medium. Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C. At 80 min the bioluminescence of cells treated with 2.3 mM menadione was 1200-fold greater than in the untreated control.

#### EXAMPLE 14

##### Response of *MicF* transformed host cell to methyl viologen or hydrogen peroxide

*E. coli* strains containing plasmids pMicFLux1 and pMicFLux2 were grown overnight at 37 °C in LB medium and diluted into the fresh LB medium and grown at 37 °C to early log-phase. 40 µL of cells were added to 60 µL of LB medium and various concentrations of methyl viologen (diluted from a 200 mg/mL stock solution in water) or hydrogen peroxide (diluted from a 0.3% solution in water). Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C. Data is shown below in Tables IX and X.

TABLE IX

Strain	Time of Max Ind.	[MV] for Max Ind.	Induction Ratio
DPD2515	120 min#	2.2 mM	99.9
DPD2516	50 min	8.75 mM*	14.7
DPD2517	120 min#	2.2 mM	1.8
DPD2518	120 min#	2.2 mM	1.4
DPD2519	120 min#	2.2 mM	87.5
DPD2520	120 min#	2.2 mM	67.2

#Longest induction time analyzed.

\*Maximum concentration tested.

TABLE X

Strain	Time of Max Ind.	[H <sub>2</sub> O <sub>2</sub> ] for Max Ind.	Induction Ratio
DPD2515	65 min	1.65 mM	47.5
DPD2516	40 min	0.41 mM	5035
DPD2517	80 min	1.65 mM	4.2
DPD2518	80 min	1.65 mM	1.6
DPD2519	60 min	1.65 mM	2.2
DPD2520	60 min	1.65 mM	2.6

EXAMPLE 15

5

Response of *UspA* transformed host cellto ethanol or copper sulfate

*E. coli* strain DE134 containing plasmid pUspALux.2 was grown overnight at 26 °C in LB medium containing Kanamycin (50 µg/mL) and diluted into the fresh LB medium containing Kanamycin (50 µg/mL) and grown at 26 °C to early log-phase. 50 µL of cells were added to 50 µL of LB medium containing Kanamycin (50 µg/mL) and various concentrations of ethanol (added directly to the medium) or copper sulfate (diluted from a 250 mM stock solution in water). Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C. The maximum response induced by ethanol was observed when the final concentration of ethanol was 4%; at 60 min after addition of ethanol the induction ratio was 148 fold. The maximum copper sulfate induction resulted when the final concentration was 5 mM; at 60 min the induction ratio was 6.9 fold.

### EXAMPLE 16

#### Response of *xthA* transformed host cell to propionate, acetate or hydrogen peroxide

*E. coli* strains containing plasmids with the *xthA* promoter fused to the *lux* operon were grown overnight at 26 °C in LB medium containing Kanamycin (25 µg/mL) and diluted into the fresh LB medium containing Kanamycin (25 µg/mL) and grown at 26 °C to early log-phase. 50 µL of cells were added to 50 µL of LB medium containing Kanamycin (25 µg/mL) and various concentrations of acetate (diluted from a 2 M stock solution in water), propionate (diluted from a 2 M stock solution in water), or hydrogen peroxide (diluted from a 30% stock solution in water). Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C. At 9 h after addition of 0.025% hydrogen peroxide to strain DPD2778, the bioluminescence was 70-fold greater than in the control with no addition; at 1 d after addition of 100 mM acetate, the induction ratio was 54; and at 3 d after addition of 100 mM propionate the response ratio was 207. For strain DPD2781, at 18 h after the addition of 0.05% hydrogen peroxide the bioluminescence was 660-fold greater than in the control with no addition; at 1 day after addition of 100 mM acetate, the induction ratio was 61; and at 3 d after addition of 100 mM propionate the induction ratio was 291.

### EXAMPLE 17

#### Response of *his* transformed host cell to gene expression

*E. coli* strains containing plasmids with the *his* promoter fused to the *lux* operon were shown to be regulated by the stringent response system in a genetic experiment showing the dependence of gene expression on the presence of the appropriate regulatory elements. Plasmid DNA was placed by CaCl<sub>2</sub> mediated transformation into otherwise isogenic strains, with normal regulation (strain CF1648), mutated in the *relA* regulatory gene (strain CF1693), or mutated in both the *relA* and *spoT* regulatory genes (strain 1651). These strains were obtained from M. Cashel (Xiao et al. (1991) Residual Guanosine 3'5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. J. Biol. Chem., 266: 5980-5990). The strains were grown in LB medium containing Ampicillin (150 µg/mL) overnight at 37 °C and diluted in the same medium and grown at 37 °C until early log-phase. Luminescence was quantitated in a Dynatech ML3000 luminometer at 26 °C. Three plasmids each demonstrated reduced bioluminescence in a *relA* mutant, and dramatically reduced bioluminescence in the *relA*, *spoT* double mutant. Data is shown below in Table XII.

TABLE XII

Plasmid	Host strain genotype	Fold reduction in bioluminescence
pHisLux5	<i>relA<sup>+</sup> spoT<sup>+</sup></i>	1X
pHisLux5	<i>relA<sup>-</sup> spoT<sup>+</sup></i>	5.7X
pHisLux5	<i>relA<sup>-</sup> spoT<sup>-</sup></i>	137X
pHisLux9	<i>relA<sup>+</sup> spoT<sup>+</sup></i>	1X
pHisLux9	<i>relA<sup>-</sup> spoT<sup>+</sup></i>	4.4X
pHisLux9	<i>relA<sup>-</sup> spoT<sup>-</sup></i>	351X
pHisLux12	<i>relA<sup>+</sup> spoT<sup>+</sup></i>	1X
pHisLux12	<i>relA<sup>-</sup> spoT<sup>+</sup></i>	4.5X
pHisLux12	<i>relA<sup>-</sup> spoT<sup>-</sup></i>	196X

- These fusions could also be induced by exposure to serine hydroxamate. This compound is a specific inhibitor of tRNA<sup>ser</sup> aminoacylation by seryl-tRNA synthetase [Pizer and Tosa (1971) J. Bacteriol. 106:972-982]. Cells were grown overnight with shaking at 29 °C in minimal E medium (Davis et al., Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1980) supplemented with uracil (25 µg/mL), kanamycin sulfate (10 µg/mL) and glucose (0.4%). The cells were diluted 20 fold in the same medium modified only by omission of kanamycin sulfate and grown as noted above to between 19 and 34 Klett Units. A 2 mg/mL solution of D,L-serine hydroxamate in water was diluted (serial 2-fold dilutions) in the same medium modified only by omission of kanamycin sulfate. These dilutions (50 µL) were mixed with 50 µL of actively growing cultures in a microtiter plate. Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C. After 1180 min of incubation the following induction ratios were observed:

TABLE XIII

Strain	Description	[Ser. Hyd.] for Max Ind.	Induction Ratio
DPD1534	pHisLux5/RFM443	250 µg/mL	>2000
DPD1535	pHisLux9/RFM443	250 µg/mL	>1000
DPD1536	pHisLux12/RFM443	250 µg/mL	>400
DPD1540	pHisLux5/DE112	250 µg/mL	9
DPD1541	pHisLux9/DE112	250 µg/mL	3
DPD1542	pHisLux12/DE112	250 µg/mL	1

This data implies that we have capitalized upon the knowledge of the stringent response mechanism to develop a biosensor capable of detecting a wide range of amino acid biosynthetic inhibitors. Since many herbicides are inhibitors of amino acid biosynthesis, this biosensor may be a useful detector of several herbicides [e.g., acetolactate synthase-directed herbicides (including those in the sulfonylurea, imidazolinone, and triazolopyrimidine classes), phosphinothricin and glyphosate].

#### EXAMPLE 18

##### Response of *phoA* transformed host cell to limiting phosphate

*E. coli* strains DPD1522 through DPD1533 containing plasmids with the *phoA* promoter fused to the *lux* operon were shown to respond to limiting phosphate. These strains were streaked for single colonies on MOPS media (Bochner et al. (1982) Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography, *J. Biol. Chem.* 257: 9759-9769) lacking tricaine and containing glucose (0.4%) as the carbon source, vitamin B1 (0.00002%), and a standard concentration of phosphate (2.0 mM) or a limiting concentration of phosphate (0.1 mM). Following overnight incubation at 37 °C, the plates were allowed to cool to room temperature and were exposed to DuPont Reflections™ film for various amounts of time. The strains growing on the standard concentration of phosphate required 3 h to result in significant exposure of the film. In contrast, the strains growing on limiting phosphate media required only 1 min to yield significant exposure of the film, thus demonstrating induction of bioluminescence by a limiting phosphate source.

This result was confirmed by an experiment conducted with a luminometer. Cultures were grown overnight with shaking at 29 °C in the above minimal medium containing 2 mM potassium phosphate supplemented with glucose (0.4%) uracil (25 µg/mL) and kanamycin sulfate (10 µg/mL). Cells were collected by centrifugation prior to resuspension in an equal volume of the same medium modified only by the omission of kanamycin sulfate and potassium phosphate. 50 µL of cells were added to 50 µL of the same medium lacking kanamycin sulfate and modified to give a final concentrations of potassium phosphate that ranged from 0-2000 uM. Light output was quantitated in a Dynatech ML3000 luminometer at 26 °C as a function of time for more than 300 min after addition of the resuspended cells. Typical results for two strains [DPD1522 (pPhoALux3/W3110) and DPD1523 (pPhoALux4/W3110)] are presented below:

TABLE XIV

Strain	uM Phosphate	Maximal Induction Ratio	Initial Induction Time (min)*
DPD1522	0	1000	20
	31	500	50
	63	400	180
	125	3	260
	250	ni	ni
	500	ni	ni
	1000	ni	ni
	2000	ni	ni
DPD1523	0	>900	20
	31	700	20
	63	400	125
	125	350	175
	250	ni	ni
	500	ni	ni
	1000	ni	ni
	2000	ni	ni

ni: induction not observed

\*time of measurable increase in luminescence over baseline reading

### EXAMPLE 19

#### Response of *glnA* transformed host cell to glutamine as a sole nitrogen source

- 5        *E. coli* strain DPD2831 was grown overnight in minimal phosphate medium (Bender et al., (1977) Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*, *J. Bacteriol.*, 129: 1001-1009) containing 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. These cultures were collected by centrifugation and resuspended in
- 10       either the same medium (control) or in that medium lacking (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, but containing 0.004% glutamine as the sole nitrogen source. Luminescence was quantitated in a Dynatech ML3000 luminometer at 26 °C. At 62 min after resuspension the cells in the medium with the poor nitrogen source (glutamine) had 62-fold greater bioluminescence than did the control culture..



EXAMPLE 20Construction of *lac* containing plasmids and host cells

*E. coli* strains were constructed such that plasmid-borne *lux* genes of *Vibrio fischeri* were under control of the *E. coli lac* promoter. A 232 basepair Pvu II to Eco RI fragment of pUC19 (Yanisch-Perron et al., (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119) was ligated into Sma I and EcoRI digested pUCD615 (Rogowsky et al., (1987) Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58, *J. Bacteriol.*, 169: 5101-5112) to yield pLacLux. This plasmid was originally isolated in *E. coli* strain XL1-Blue (Bullock et al. (1987), XL1-Blue: A high efficiency plasmid transforming *recA* *Escherichia coli* strain with beta-galactosidase selection, *Biotechniques*, 4: 376-379) which contains an F'*lacI<sup>q</sup>*, so that the *lux* genes were inducible by IPTG. The plasmid was also placed by CaCl<sub>2</sub> mediated transformation into *E. coli* strains W3110, RFM443, and DE112 (see Table V, Example 10).

EXAMPLE 21Response of *lac* transformed host cell  
to carbon source levels

Glucose is the preferred carbon source for *E. coli*. *E. coli* strains TV1058 and TV1068 each containing the plasmid pLacLux were grown overnight in LB medium containing kanamycin (25 µg/mL) either lacking or containing 0.4% glucose at 26 °C. The overnight cultures were diluted and grown to early log phase in the same media as the overnight culture. Culture turbidity was measured with a Klett-Summerson colorimeter with a #66 red filter. Luminescence present in 50 µL of cell culture was quantitated in a Dynatech ML3000 luminometer at 26 °C. Data are given below in Table XIV.

TABLE XV

Strain	Media	Klett Units	RLU/50 µL cells
TV1058	-glucose	18	32.4
TV1058	+glucose	22	0.087
TV1068	-glucose	21	29.1
TV1068	+glucose	21	0.084

Thus, cells containing the plasmid pLacLux increase bioluminescence when grown on the suboptimal carbon sources present in LB medium.

## EXAMPLE 22

### Construction and Response of *fabA* transformed host cell to fatty acid starvation

The *fabA* gene encodes the enzyme responsible for the placement of a double bond in the fatty acids and hence membrane of *E. coli*. Such double bonds are an absolute requirement for growth. Synthesis of *fabA* is directed by two promoter elements: a low level, constitutive upstream and an inducible downstream promoter. The location of the two promoters in the sequence surrounding *fabA* has been determined. The PCR primers shown in Table IV are designed to allow cloning of the inducible downstream promoter without the constitutive upstream promoter. The transcription of the downstream promoter can be modulated at least 10 fold at the RNA level (Henry et al., *J. Mol. Biol.* 222:843-849 (1991)). Control of the dual *fabA* promoters, studied in *fabA-lac* fusions, has shown a 13 fold modulation at the level of  $\beta$ -galactosidase specific activity (Henry et al., *Cell*, 70: 671-679 (1992)). Control of *fabA* expression is mediated by the *fadR* gene product (Nunn et al., *J. Bacteriol.* 154:554-560 (1983)). The FadR protein stimulates *fabA* transcription by binding to the -40 region of the regulated, downstream *fabA* promoter. If there is an excess of membrane synthetic capacity, long-chained acyl-CoA molecules accumulate. These molecules bind to the FadR protein, dissociating it from the regulated *fabA* promoter (Henry et al., *Cell*, 70: 671-679 (1992)). The *fabA-lux* fusion is thus expected to serve as a monitor of the state of membrane synthesis. Under conditions of fatty acid starvation, long chain acyl-CoA pools will be low and expression of *fabA-lux* should be high; excess fatty acids should result in large pools of long chain acyl-CoAs and hence low levels of *lux* expression from the fusion. This fusion should monitor not only fatty acid synthetic inhibition but CoA availability, which can be limited by many factors including inhibition of the isoleucine-valine synthetic enzyme acetolactate synthase (LaRossa et al., pp. 108-121 in *Biosynthesis of Branched Chain Amino Acids*, ed. by Barak, Chipman and Schloss, VCH Publishers, New York, 1990). Methods of producing the stress of fatty acid starvation on a potential detector organism containing a *fabA::lux* fusion might include inhibition of fatty acid desaturation by inclusion of 3-decenoyl-N-acetylcysteamine in the growth medium (Nunn et al., (1983) *J. Bacteriol.* 154:554-560) or the sequestering of intracellular CoA as propionyl-CoA by the action of the herbicides such as sulfometuron methyl (Van Dyk et al., *Mol Gen Genet* (1987) 207:435-440) or the amino acid valine (LaRossa et al., (1987) *J. Bacteriol.* 169:1372-1378).

### Construction of *fabALux* and transformation of RFM443

Construction of a transforming plasmid containing the *fabA::lux* gene fusion is prepared using methods and materials essentially as described in

Example 3 for the preparation of pRY001 and pRY002. PCR amplification of the *fabA* promoter is accomplished with the primers listed in Table IV. The sequence of the *fabA* gene is known and is readily available from the Genbank database of nucleic acid sequences. The plasmid carrying the *fabA::lux* fusion is referred to as pFabALux.

*E. coli* host RFM443 is transformed with the pFabALux in using the materials and methods described for the construction of WM1021 and WM1202 in Example 3.

*E. coli* host RFM443 is transformed with the pFabALux is grown overnight in minimal E medium containing kanamycin (10 µg/mL) at 29 °C. The overnight cultures are diluted and grown to early log phase in the same media as the overnight culture. Culture turbidity is measured with a Klett-Summerson colorimeter with a #66 red filter. Luminescence present in 50 µL of cell culture in the presence or absence of 50 µg/mL of sulfometuron methyl is quantitated in a Dynatech ML3000 luminometer at 26 °C. It is seen that cultures in the presence of sulfometuron methyl demonstrate a 10-25 fold increase in luminesces when compared with cultures in the absence of sulfometuron methyl.

Thus, cells containing the plasmid pFabALux are expected to increase bioluminescence when grown under conditions of fatty acid synthesis inhibition.

#### EXAMPLE 23

##### Responses to a Physical Challenge

The *recA::lux*, *uvrA::lux*, *grpE::lux*, *dnaK::lux*, *katG::lux*, *micF::lux* and *uspA::lux* fusions were exposed to ultraviolet light irradiation. All but *grpE::lux* responded to this physical challenge by increasing bioluminescence. Strains were grown overnight with shaking in LB medium supplemented with kanamycin sulfate (25 µg/mL) at 26 °C. After 20-fold dilution into LB medium, culture was continued with shaking at 26 °C until densities of 20-40 Klett units were reached. Cultures (50 µl) and fresh LB medium (50 µl) were added to wells of a microtiter plate prior to irradiation at 254 nm with a Stratalinker 1800 instrument (Stratagene). Subsequently, light output was quantitated as a function of time after irradiation in a Dynatech ML3000 luminometer at 26 °C. Response ratios were calculated after 240 min of incubation. They are reported in the Table XVI:

TABLE XVI

Response Ratio:

Dose (mjoule/cm <sup>2</sup> )	DPD2794 (pRecAlux3/ RFM443)	DPD2815 pUvrAlux2/ W3110	TV1061 (pGrpElux.S/ RFM443)	WM1202 [pRY002/RFM443 dnaK fusion)	DPD2507 (pKatGLux2/ W3110)	DPD2515 (pMicFLux1/ W3110)	DE158 (pUspALux13/ RFM443)
0	=1	=1	=1	=1	=1	=1	=1
0.1	1	1	1	1	1	1	1
0.4	2	2	1	2	1	1	1
2	5	14	1.2	5	1.2	1.5	2
10	10	29	1.5	20	2	4	2.5
50	15	57	1.5	30	2.5	7	3
250	15	63	<1	25	3	10	5
1250	19	76	<1	20	2.5	10	8

EXAMPLE 24Small Volume Assay

Example 24 demonstrate that the detector cells of the present invention may be adapted to assay methods utilizing as little as 0.5ul of assay volume with reproducible results.

Experiment 1

An overnight culture of TV1061 was grown in LB-100 µg/ml Ampicillin. The following day the culture was diluted 1/50 and allowed to grow to an OD600 of 0.8. 7 microliters of cells were added to each of 60 wells of a microtiter plate with wells 3.5 mm in diameter and 0.9 mm deep, and 8 microliter volume. 1.2 microliters of 20% ethanol was added to half the wells, to give a final concentration of 3%.

After 40 min at room temperature, an X-ray film was placed on top of the sheet for 10 min. 1 microliter was sampled from two different wells at times 0, 30, and 60 minutes after ethanol addition. Serial dilutions were made and plated onto LB-Ampicillin plates. The X-ray film was developed.

The next day, colonies on plates were counted, and the concentration of cells determined to be  $3.9 \times 10^8$  ml, or  $2.7 \times 10^6$  cells per well. The X-ray film indicated a clear induction of luminescence by ethanol. During the 1 h course of the experiment the cell density increased 2.2 fold.

Experiment 2

An overnight culture of TV1061 was grown in LB-100 µg/ml Ampicillin. The culture was diluted 1/50 and allowed to grow to an OD600 of 0.5. Cells were split into 6 tubes with the following dilutions and additions:

- |    |        |  |
|----|--------|--|
| 25 | Tube 1 | 500 µl + 15 µl 100% EtOH                     |
|    | Tube 2 | 500 µl                                       |
|    | Tube 3 | 500 µl of a 1/20 dilution + 15 µl 100% EtOH  |
|    | Tube 4 | 500 µl of a 1/20 dilution                    |
|    | Tube 5 | 500 µl of a 1/100 dilution + 15 µl 100% EtOH |
| 30 | Tube 6 | 500 µl of a 1/100 dilution                   |

The dilutions were taken to the dark room, where the induction of luminescence by ethanol was clear in the undiluted and 1/20 dilution samples.

Three samples of 1 microliter each were removed from Tubes 1-6 and placed on ice. 8, 2, and 0.5 microliter aliquots were added to each of 9 wells of a microtiter plate with 8 microliter volume wells. X-ray films were placed on top of the microtiter plate for 15, 30, and 90 min. Serial dilutions were made of the samples placed on ice, and plated onto LB-Ampicillin plates. Colonies were counted the next day, and the original cell concentration determined to be  $1.2 \times 10^8$  cells/ml.

- As determined by the X-ray films, good induction by ethanol occurred in all undiluted samples, including the 0.5 microliter sample, which contained  $6.0 \times 10^4$  cells. Of the 1/20 dilution, induction was clearly observed in the 8 microliter sample. These results indicate that the system was not stressed by low well
- 5 volume or by low numbers of cell.

It is apparent that all but one of the tested constructs responded to physical as well as chemical stresses.

SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT:
  - (A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
  - (B) STREET: 1007 MARKET STREET
  - (C) CITY: WILMINGTON
  - (D) STATE: DELAWARE
  - (E) COUNTRY: UNITED STATES OF AMERICA
  - (F) POSTAL CODE (ZIP): 19898
  - (G) TELEPHONE: 302-892-8112
  - (H) TELEFAX: 302-773-0164
  - (I) TELEX: 6717325
- (ii) TITLE OF INVENTION: A SMALL VOLUME, HIGHLY  
SENSITIVE METHOD FOR DETECTING  
ENVIRONMENTAL INSULTS
- (iii) NUMBER OF SEQUENCES: 24
- (iv) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
  - (B) COMPUTER: IBM PC COMPATIBLE
  - (C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
  - (D) SOFTWARE: MICROSOFT WORD VERSION 7.0A
- (v) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 60/031272
  - (B) FILING DATE: 15 NOVEMBER 1996
- (vii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: LINDA AXAMETHY FLOYD
  - (B) REGISTRATION NUMBER: 33,692
  - (C) REFERENCE/DOCKET NUMBER: CL-1015-P1

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTTAGCGGAT CCAAAGCAC AAAAAAT

27

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCAGTGAAT TCCATCTAAA CGTCTCCA

28

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACTTAAGGAT CCAAGCGATG GCGCGTAAAA

30

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCAGCGAAT TCATCGCCGC TTCCAGACAA

30



## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACTTAAGGAT CCAGAGAAGC CTGTCGGCAC

30

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCTTTGAAT TCCGCTTCTG TTTGTTTT

28

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACTTTTGGAT CCGTGTAAC GCGCGATTG

29

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCAGCGAAT TCTTCCCGGA TTAAACGCTT

30

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACTTAAGGAT CCCGAAATGA GGGCGGGAAA

30

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCAGCGAAT TCGAACGTTG CTGACCACGA

30

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTTAAGGAT CCCCCAAAA ATGCAGAATA

30

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCAGCGAAT TCGGGCATCC GGTGAAATA G

31

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTTAAGGAT CCGCCATTAC GTTGGCTGAA

30

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCAGCGAAT TCCCACCCGT TTCGGTCATT

30

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ACTTAAGGAT CCCTCCCGAT ACGCTGCCA

29

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGCAGCGAAT TCGGCGATGA GAATGTGTTT AT

32

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACTTAAGGAT CCAATTACTG CGCCATTCTG

30

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACATCGGAAT TCTCATAGTC GCTGCCATTT

30

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ACTTAAGGAT CCCTAATTGT ACGCATGTCA

30

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCAGCGAAT TCAAAGTCTC TGTGAATGTT

30

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACTTAAGGAT CCAGATTATC GTCAGTCAA

30

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCAGCGAAT TCGGCCAATC AGCAAAATAA

30

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACTTTCGGAT CCTTGGTGCA ACATTCACAT

30

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCAGCGAAT TCTCAGCGGA CATCGTCAGT

30

Applicant's or agent's file reference number	SL1015	International application	
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(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>6 and 7</u> , line s <u>1 and 2</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 13 May 1993 (13.05.93)	Accession Number 69315
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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Applicant's or agent's file reference number	1015	International application:
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Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 13 May 1993 (13.05.93)	Accession Number 69314
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's file reference number	JL1015	International application.
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A. The indications made below relate to the microorganism referred to in the description on page <u>6 and 7</u> , lines <u>1 and 2</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 13 May 1993 (13.05.93)	Accession Number 69313
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
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Applicant's or agent's file reference number	LL1015	International application.
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A. The indications made below relate to the microorganism referred to in the description on page <u>6 and 7</u> , line s <u>1 and 2</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 13 May 1993 (13.05.93)	Accession Number 69316
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

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Applicant's or agent's file reference number	JL1015	International application:
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A. The indications made below relate to the microorganism referred to in the description on page <u>6 and 7</u> , line s <u>1 and 2</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 03 December 1992 (03.12.92)	Accession Number 69142
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
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In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>6</u> and <u>7</u> , line s <u>1</u> and <u>2</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 US	
Date of deposit 03 December 1992 (03.12.92)	Accession Number 69141
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

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A. The indications made below relate to the microorganism referred to in the description on page <u>6 and 7</u> , line s <u>1 and 2</u>	
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Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
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C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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We claim:

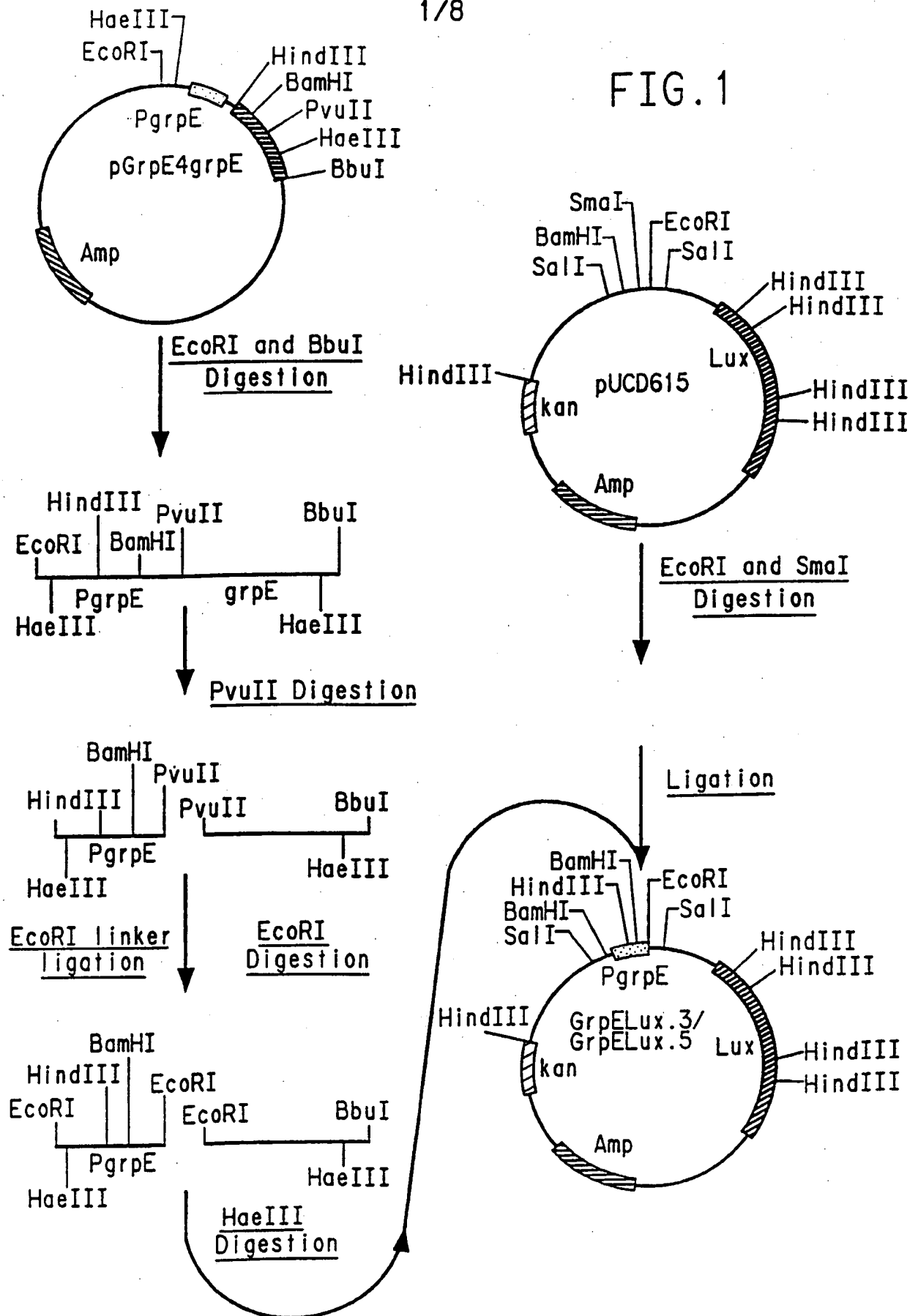
1. An improved method of detecting environmental stress on a microorganism population, of the steps (a) exposing a prokaryote detector organism to an environmental insult, said prokaryote detector organism being genetically engineered to contain an expressible heterologous *luxCDABE* gene complex under the control of a stress inducible promoter sequence wherein said promoter is responsive to a global regulatory circuit; and (b) measuring an increase in luminescence of said prokaryote detector organism, the improvement comprising
  - (a) dispensing a medium containing a prokaryote detector organism into at least one container having a volume of about 0.5 ul to about 10 ul, said prokaryote detector organism being genetically engineered to contain an expressible heterologous *luxCDABE* gene complex under the control of a stress-inducible promoter sequence wherein the promoter is responsive to a global regulatory circuit;
  - (b) exposing the prokaryote detector organism to an environmental insult;
  - (c) using a luminescent detector to measure an increase in luminescence of the prokaryote detector organism.
2. The method of Claim 1 wherein the container is a well of a microtiter plate and the luminescent detector is an x-ray film.
3. The method of Claim 1 wherein the prokaryote detector organism is in the log phase when exposed to the environmental insult.
4. The improved method of Claim 1 wherein the stress inducible promoter sequence is selected from the group consisting of *groEL*, *dnaK*, *grpE*, *phoA*, *glnA*, *lon*, *lysU*, *rpoD*, *clpB*, *clpP*, *uspA*, *katG*, *uvrA*, *frdA*, *micF*, *fabA*, *lac*, *his*, *sodA*, *sodB*, *soi-28*, *recA*, *xthA*, and *narG*.
5. The method of Claim 1 wherein the prokaryote detector organism is an enteric prokaryote.
6. The method of Claim 1 wherein the prokaryote detector organism is *E. coli*.
7. The method of Claim 1 further comprising correlating the increase in luminescence to the level of environmental stress.
8. The method of Claim 1 wherein the environmental insult is selected from the group consisting of atrazine, benzene, copper sulfate, 2,4-dichlorophenoxy acetic acid, ethanol, methanol, 2-nitrophenol, 4-nitrophenol, pentachlorophenol, phenol, toluene, dimethylsulfoxide, lead nitrate, cadmium chloride, sodium chloride, menadione, ethidium bromide, serine hydroxamate, acetate, propionate, hydrogen peroxide, puromycin, mercury chloride, 2,4-

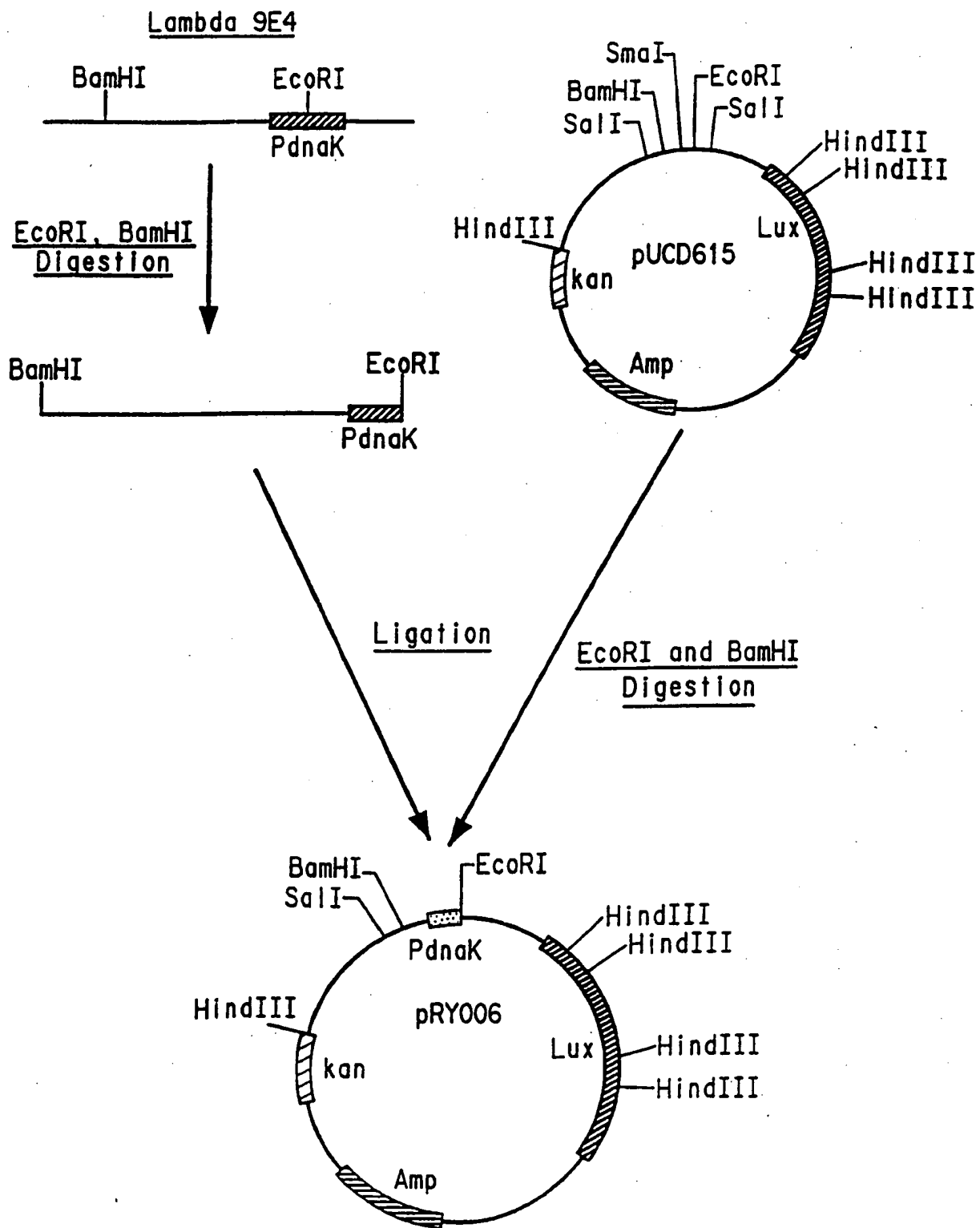
dichloroaniline, propanol, butanol, isopropanol, methylene chloride, Triton X100, acrylamide, methyl viologen, mitomycin C, xylene, and ultraviolet irradiation.

9. The method of Claim 1 wherein the environmental insult stimulates
- (i) protein damage that alters the action of *rpoH* or its gene product,
  - (ii) oxidative damage that alters the action of *oxyR* or its gene product,
  - (iii) oxidative damage that alters the action of *soxRS* or their respective gene products,
  - (iv) membrane damage that alters the action of *fadR* or its gene product,
  - (v) amino acid starvation that alters the action *relA* and *spoT* or their respective gene products,
  - (vi) carbon starvation that alters the action of *cya* and *crp* or their respective gene products,
  - (vii) phosphate starvation that alters the action of *phoB*, *phoM*, *phoR*, and *phoU* or their respective gene products;
  - (viii) nitrogen starvation that alters the action of *glnB*, *glnD*, *glnG*, and *glnL* or their respective gene products;
  - (ix) the universal stress response that alters the action of its regulators;
  - (x) the stationary phase response that alters the action of *rpoS* or its gene product; or
  - (xi) DNA damage that alters the action of *lexA* or *recA* or their respective gene products.

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FIG. 1

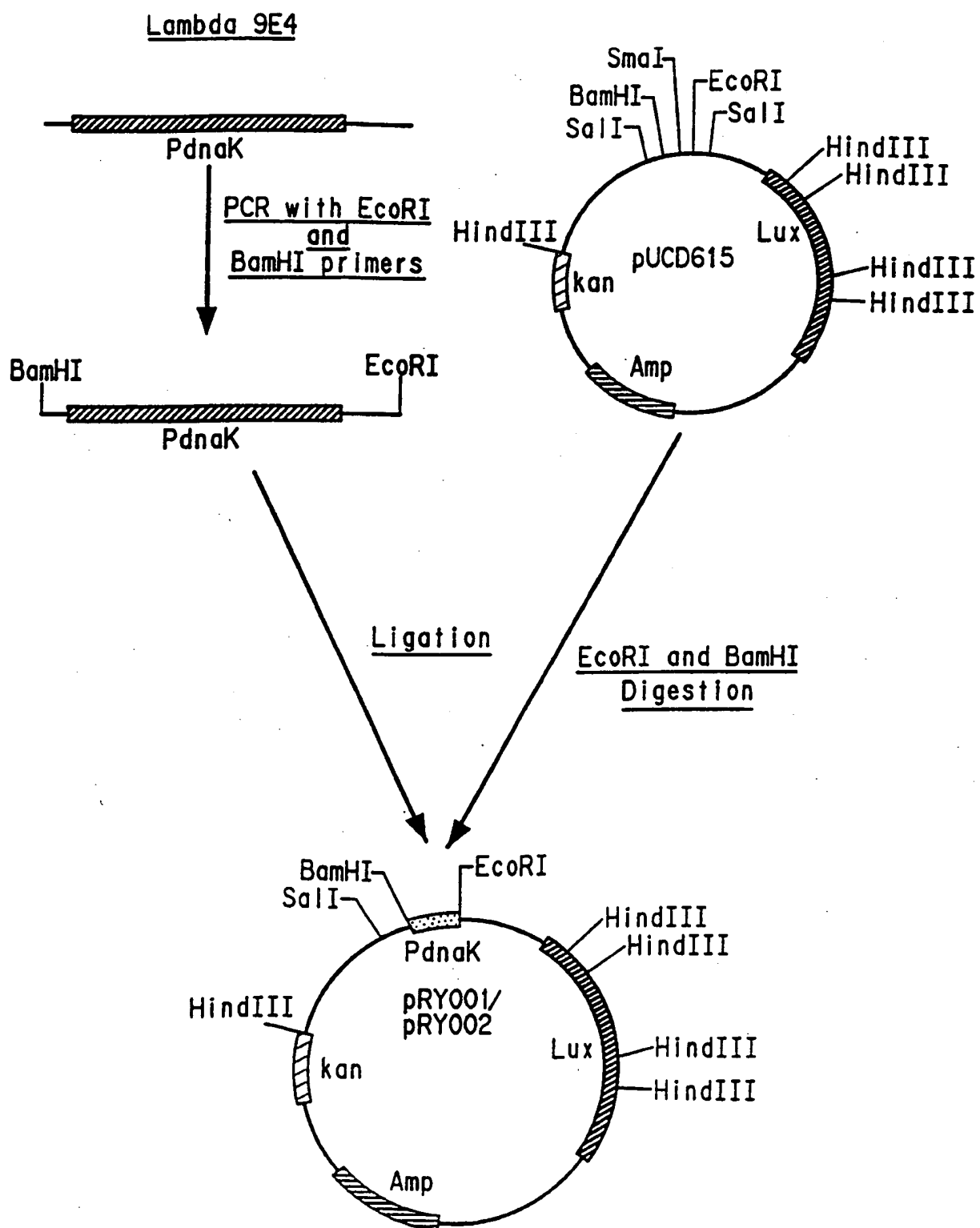


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FIG. 2



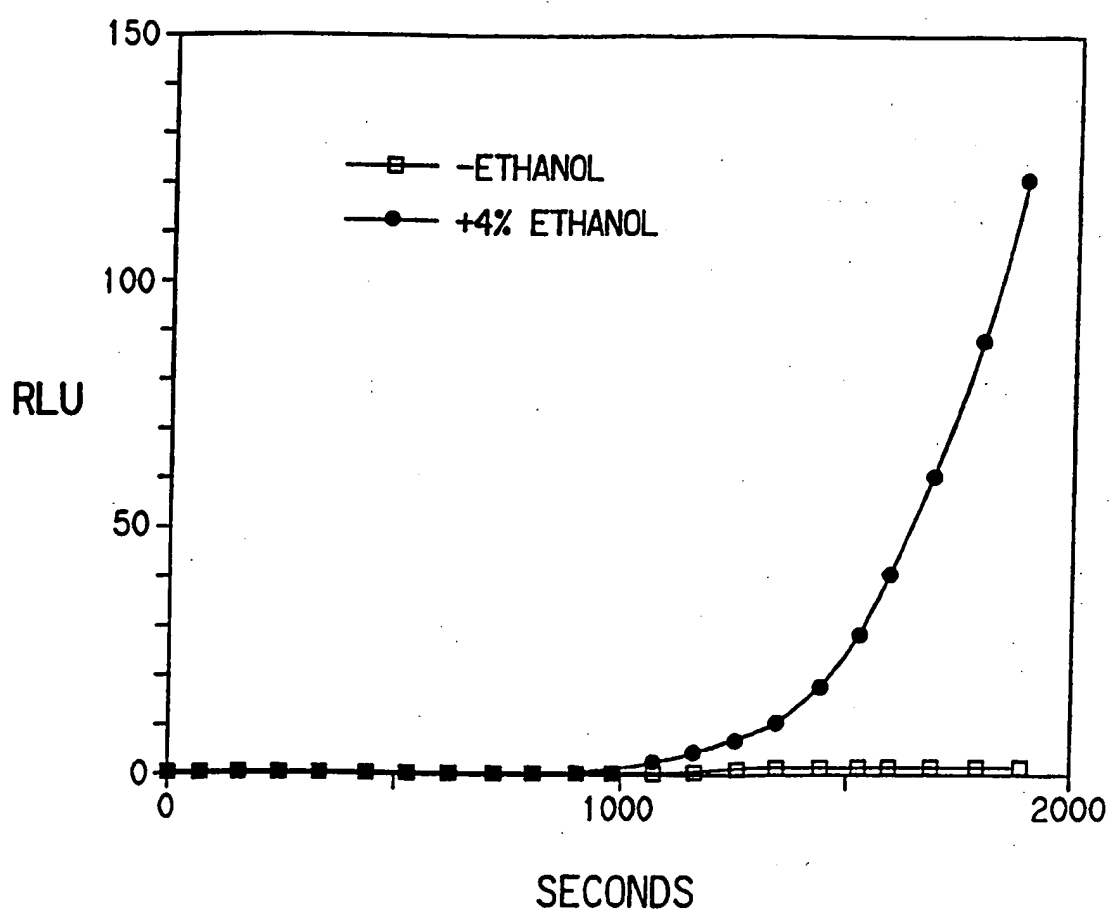
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FIG. 3



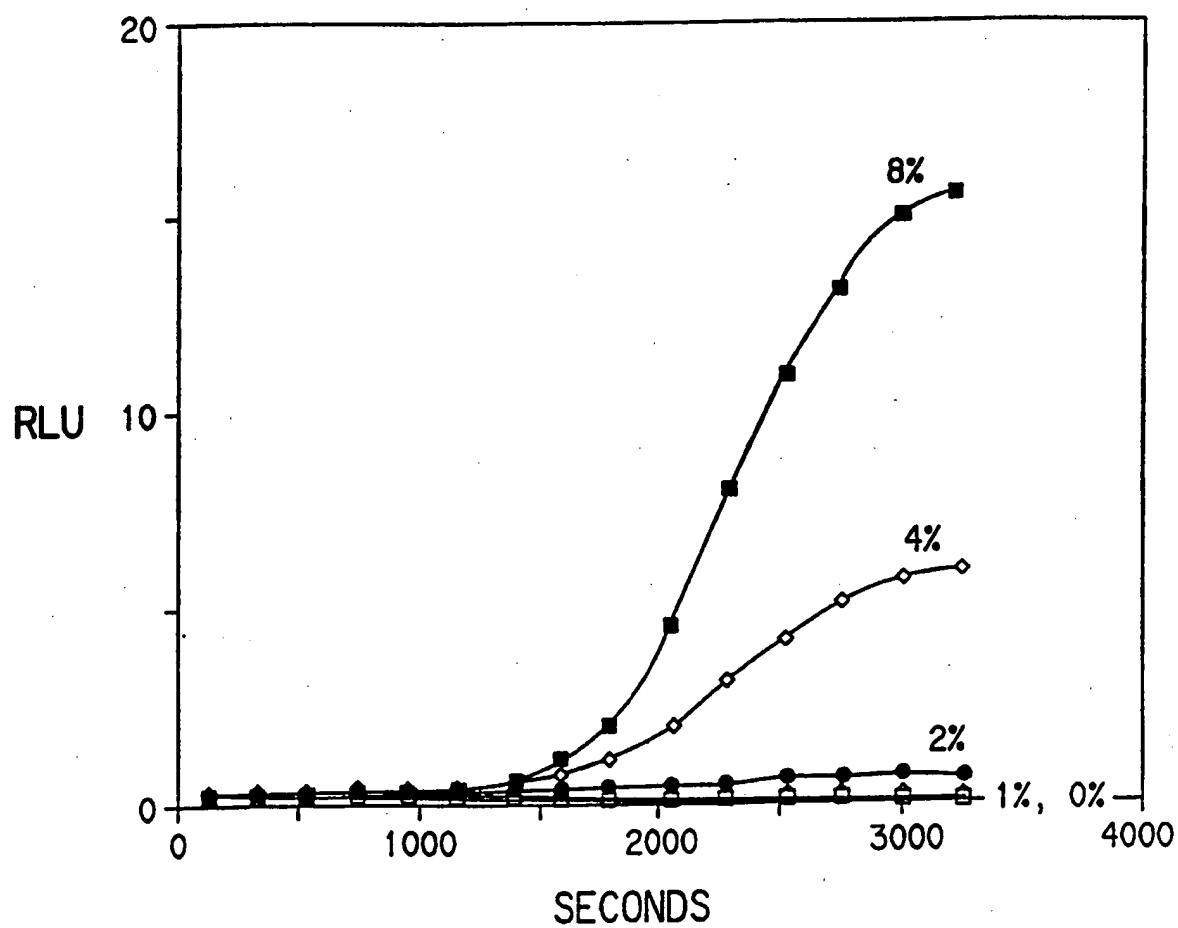
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FIG. 4



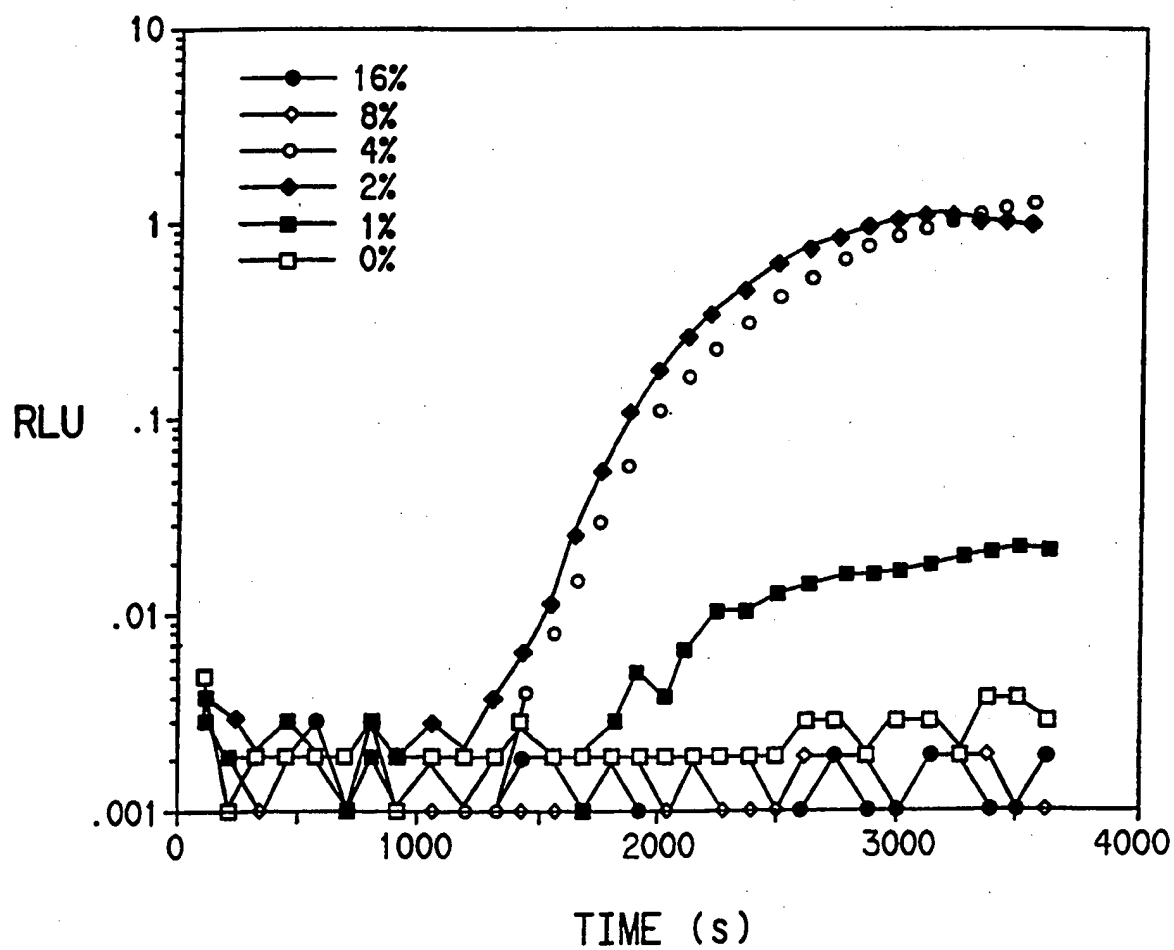
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FIG. 5



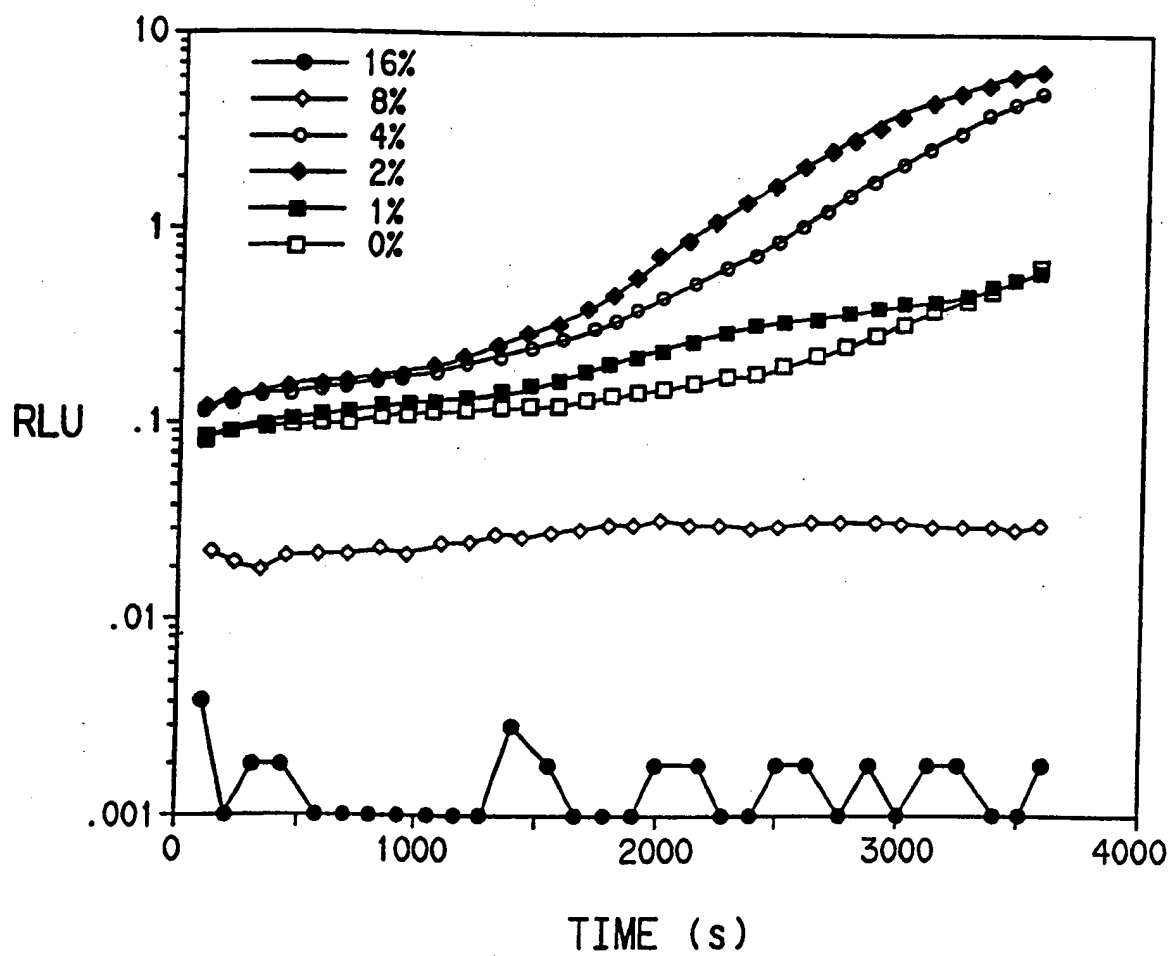
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FIG. 6a



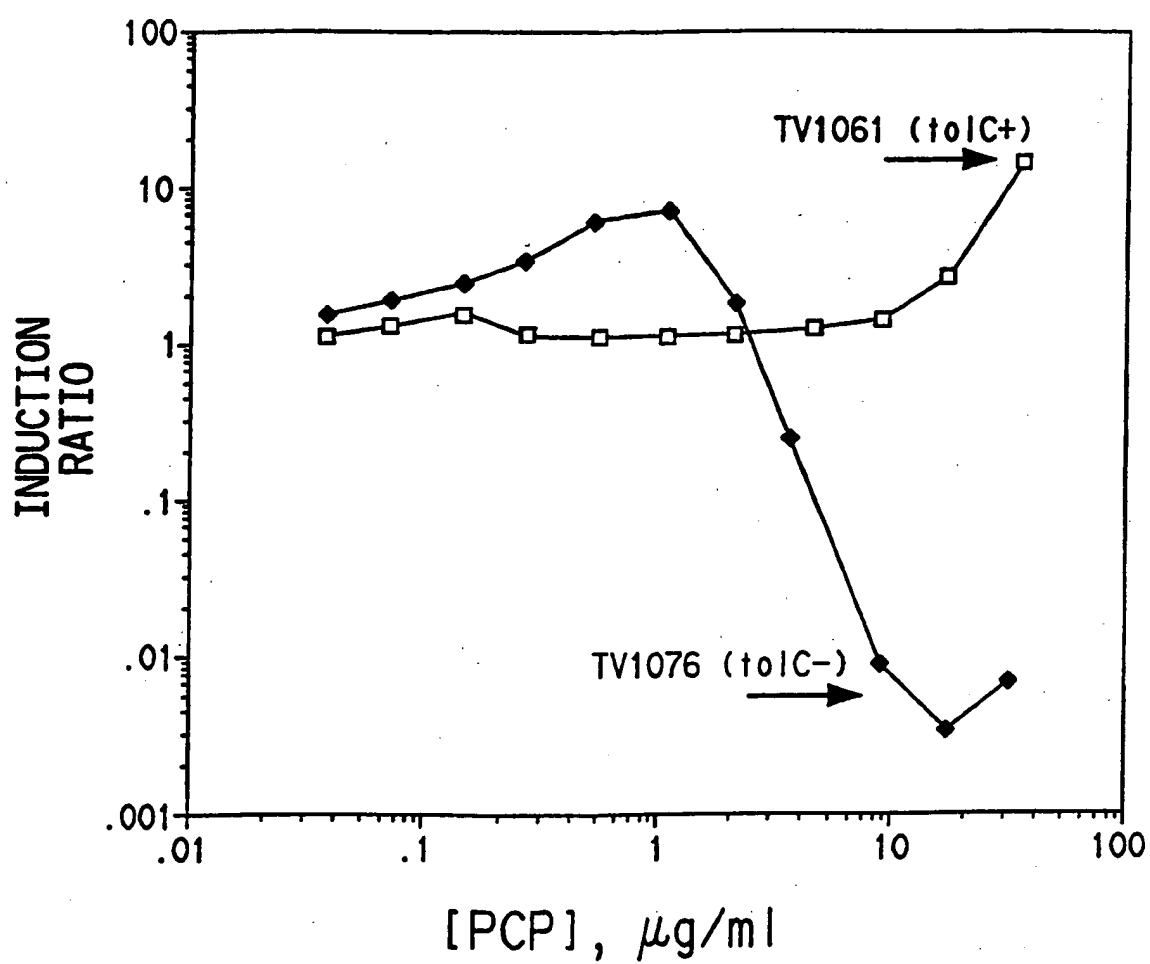
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FIG. 6b



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FIG. 7



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/20793

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/70 C12Q1/02 C12Q1/68 C12Q1/66

According to International Patent Classification(IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13831 A (DU PONT ; LAROSSA ROBERT ALAN (US); MAJARIAN WILLIAM ROBERT (US); D) 23 June 1994 cited in the application see the whole document ---	1-9
X	WO 96 16187 A (DU PONT) 30 May 1996 see the whole document ---	1-9
X	WO 94 01584 A (HARVARD COLLEGE ; FARR SPENCER B (US)) 20 January 1994 see the whole document ---	1-9
A	EP 0 649 905 A (TOYODA CHUO KENKYUSHO KK) 26 April 1995 see the whole document -----	1-9

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Further documents are listed in the continuation of box C.

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Patent family members are listed in annex.

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Date of the actual completion of the international search

30 March 1998

Date of mailing of the international search report

06/04/1998

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Hagenmaier, S

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information on patent family members

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